EPSTEIN-BARR VIRUS INFECTION IN CARDIOTHORACIC TRANSPLANT RECIPIENTS

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A thesis submitted to the University of Edinburgh for the degree of Doctor of Philosophy
2000

Declaration of authorship

This thesis was composed by the undersigned candidate. The work included is the candidates own with contributions from other researchers as described in the acknowledgments section.

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Abstract

Epstein-Barr virus (EBV) is a human DNA herpesvirus which establishes a persistent latent infection *in vivo* in B-lymphocytes. Iatrogenic immunosuppression to prevent rejection of transplanted organs predisposes to EBV positive post-transplant B-lymphoproliferative disease (PTLD).

This study followed 96 adult cardiothoracic transplant recipients for up to 1110 days (mean follow up time of 415 days) post-transplant. EBV load in peripheral blood mononuclear cells (PBMs) was determined in each sample using a sensitive semi-quantitative DNA PCR. RT-PCRs were developed to examine whether the pattern of EBV gene expression changed following transplantation due to immunosuppressive therapy and how these changes relate to PTLD development.

The results show that post-transplant EBV load significantly increases above that detected in normal EBV seropositives in 55% of transplant recipients and reaches levels equivalent to that detected in other patients with PTLD in 14% of patients, and equivalent to patients with IM in 40%. Thus high EBV load is associated with, but not diagnostic of EBV associated disease.

Following transplantation EBV genes are expressed which are not detected in normal EBV carriers which may be responsible for increased EBV load through a combination of lytic replication and EBV-driven B-lymphoproliferation. The highest EBV loads detected were in samples with lytic replication and unrestricted latent gene expression. Despite detection of patterns of EBV gene expression and levels of EBV equivalent to that in patients with PTLD, these patients did not develop apparent disease. Therefore despite profound immunosuppression, and partial loss of control of EBV infection following transplantation, PTLD did not develop. These results suggest that additional factors are required for PTLD development.

Acknowledgements

I would like to thank Professor Dorothy H Crawford for her invaluable guidance and support. I would also like to thank Dr Louise Brooks for her supervision and advice and Dr Alero Thomas for helpful discussion and for providing PTLD samples. I am extremely grateful to our collaborators at Harefield Hospital, Middlesex: Professor Sir Magdi Yacoub, Dr Beverley Hunt, Miss Rachael Parratt and colleagues for their crucial role in this study through providing clinical samples and patient treatment details. Also Dr Peter Amlot of the Royal Free Hospital, London, for providing PBM samples from patients with PTLD. Also Professor Gordon Murray for his advice on the statistical analysis used in this study. I am grateful to Maria Bokhari for her technical assistance in completing Southern blots and EBV serological analysis during the final stages of this study, and to Dr Ingolfur Johannessen for providing SCID mouse tumour material.

I also wish to thank my colleagues in the Department of Medical Microbiology of the University of Edinburgh for their friendship and for valuable discussions.

I am particularly indebted to my mother, father and sister to whom this thesis is dedicated and to Lara for her incredible patience and caring support.

This study was funded by the Medical Research Council.

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Abbreviations and symbols

aBL	AIDS related Burkitt's lymphoma
Abs	Antibodies
ACV	Acyclovir
AIDS	Acquired immunodeficiency syndrome
ALG	Anti-lymphocyte globulin
ANOVA	Analysis of variance
Aza	Azathioprine
BL	Burkitt's lymphoma
BLCL	B lymphoblastoid cell line
BLPD	B cell lymphoproliferative disease
bp	Base pair
Bq	Becquerel
CAM	Cellular adhesion molecule
CD	Cluster designation
cDNA	Complementary DNA
Ci	Curie
CMV	Cytomegalovirus
CNS	Central nervous system
CsA	Cyclosporin A
CTLs	Cytotoxic T-lymphophocytes
Da	Daltons
dATP	Deoxyadenosine 5'-triphospate
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide 5'-triphospates
ds	Double-stranded
EA	Early antigen
EBER	EBV-encoded small RNA
eBL	Endemic Burkitt's lymphoma
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus

EDTA	Ethylene diamine-tetraacetic acid
ELISA	Enzyme-linked immunoabsorbent assay
EtBr	Ethidium bromide
FCS	Foetal calf serum
g	G-number/ gram
gp	Glycoprotein
GVHD	Graft-versus-host disease
HCl	Hydrochloric acid
HD	Hodgkin's disease
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Infectious mononucleosis
IR	Internal repeat
ISH	*In-situ* hybridisation
iv	Intravenous
K	Kilo (10^3)
KSHV	Kaposi's sarcoma-associated herpesvirus
L	Litre
LMP	Latent membrane protein
LP	Leader protein
LPD	Lymphoproliferative disease
m	Milli (10^3)
M	Molar (moles/L)/ Mega (10^6)
MA	Membrane antigen
MHC	Major histocompatibility complex
mRNA	Messenger RNA
n	Nano (10^{-9})
NHL	Non-Hodgkin's lymphoma
NK	Natural killer cell

NPC	Nasopharyngeal carcinoma
OD	Optical density
OHL	Oral hairy leukoplakia
ORF	Open reading frame
*ori*Lyt	Lytic origin of replication (lytic cycle)
*ori*P	Plasmid origin of replication (latent cycle)
p	Pico (10^{-12})
PBMC/ PBMs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Revolutions per minute
RT	Reverse transcription
sAg	Surface antigen
sBL	Sporadic Burkitt's lymphoma
SCID	Severe combined immunodeficient
SDS	Sodium dodecyl sulphate
sIg	Serum immunoglobulin
ss	Single stranded
SSC	Standard sodium citrate
TE	Tris/ EDTA
Th	T helper
TNF	Tumour necrosis factor
TP	Terminal protein
TR	Terminal repeat
Tris	Tris(hydroxymethyl)aminomethane
UL	Unique long
US	Unique short
v/v	Volume/ volume
VCA	Viral capsid antigen
VZV	Varicella Zoster virus
w/v	Weight/ volume

XLA	X-linked agammaglobulinaemia
X-LPS	X-linked lymphoproliferative syndrome
μ	Micro (10^{-3})
α	Alpha
β	Beta
γ	Gamma
ϕ	Phi
1^{o}	Primary
2^{o}	Secondary
~	Approximately
>/ ≥	Greater than/ greater than or equal to
</ ≤	Less than/ less than or equal to

1 Introduction

1.1 Organ Transplantation

The development of tissue and organ transplantation has provided enormous benefits both in patient treatment and advances in immunology. The idea of grafting parts of the body is not new; there is archaeological evidence that teeth were transplanted in man in ancient Egypt, Greece, North and South America, Italy and China (Peer 1955).

Autotransplantation (where the donor and recipient are the same individual) of a kidney in a dog was successfully performed for the first time in 1908 (Carel 1908). In this operation, both kidneys were removed, and one replaced, following which it functioned entirely normally demonstrating that surgical interruption of the kidneys circulation and suture of the blood vessels and the ureter was possible.

It was realised that while autotransplantation was nearly always successful, allogeneic transplantation (where the donor and recipient are genetically dissimilar individuals of the same species), whilst often initially apparently successful, was nearly always ultimately a failure. Allogeneic transplantation resulted in correct function of the grafted organ for the first few days, but after six or seven days the kidneys became congested and after seven or eight months they were found to be sclerotic with a leucocyte infiltrate (Carel 1908). It was soon realised that loss of the grafted organ was not due to avascular necrosis, infarction, infection or inflammation, and the term 'rejection' was used to describe the process by which the host animal was unable to accept the transplanted organ.

Progress in understanding the immunological basis of rejection led to the development of immunosuppression techniques, including the use of whole-body irradiation and irradiation of the graft. By 1962, all transplants between unrelated individuals were performed under the influence of immunosuppressive drugs, initially 6-mercaptopurine and subsequently a derivative of it, azathioprine (Schwartz *et al* 1959, Murray *et al* 1963). Azathioprine inhibits DNA and RNA synthesis, but also incorporates into DNA, resulting in chromosomal breaks (Bach 1975), which

may in part explain why azathioprine recipients are at increased risk of developing cutaneous malignancies. Initially the standard immunosuppressive regime for transplantation was combined high doses of azathioprine and the corticosteroid prednisolone. It was soon established that greatly reduced doses of steroid were associated with fewer side effects and good graft outcome (McGeown *et al* 1980, d'Apice *et al* 1984). The great breakthrough in immunosuppressive therapy came with the development of cyclosporine (CsA), a fungal decapeptide discovered in 1976 (Borel *et al* 1976), which became accepted as the standard primary immunosuppressive therapy by the early 1980's. CsA prevents proliferation and clonal expansion of T-lymphocytes through inhibiting the effects of interleukin2 (IL2), which reduced acute rejection and gave a 10-20% improvement in one-year graft survival (European Multicentre Trial Group 1983).

New immunosuppressive drugs have been developed recently which are more specific and potent than CsA. FK506, or tacrolimus, is an inhibitor of IL2 production, and has wide ranging effects on the immune system (Goto *et al* 1991). Rapamicin is structurally similar to FK506, but antagonises the interaction of IL2 with its receptor, preventing cytokine dependent T-lymphocyte proliferation 100 times more potently than CsA (Dumont *et al* 1990). B- and T- lymphocytes, unlike other cells, rely on *de novo* purine and pyrimidine synthesis. Mycophenylate mofetil and Leflunomide inhibit purine or pyrimidine synthesis respectively, specifically inhibiting activation of lymphoid cells. Advances have also been made using anti-T-lymphocyte monoclonal (such as OKT3) or oligoclonal antibodies (such as anti-lymphocyte globulin (ALG)) to treat acute rejection (Kreis & Goldstein 1985).

The development of organ transplantation has been accompanied by realisation of numerous complications. The intensive post-operative care required means that patients are initially at risk from opportunistic and nosocomial diseases common after any major surgery. However, since these patients are subject to severe immunosuppression to prevent graft rejection, in particular, specific inhibition of their cytotoxic T-cell responses, they may be unable to control infections which would not cause severe disease in an immunocompetent individual. Transplant recipients are at risk of developing bacterial infections including *Mycobacterium*

tuberculosis, the majority of which occurs from reactivation of a latent infection, with a frequency of 0.8 - 3% in renal transplant recipients, compared with 0.1% in the general population or with *Listeria monocytogenes* (Delaney *et al* 1993, Quinbi *et al* 1990). Immunosuppressed transplant recipients are also at increased risk of fungal infection particularly in the first two months post-transplant with high mortality if untreated (Paya 1993a). Over 80% of cases are caused by *Candida albicans* or *Aspergillus sp.* (Weiland *et al* 1983). *Pneumocystis carenii* is the most common parasitic infection in transplant recipients, and is particularly prevalent as a cause of pneumonia following lung transplantation (Nicholson & Johnson 1994, Dummer *et al* 1986).

Viral disease following organ transplantation may be due to primary infection or reactivation of a latent or subclinical infection and tend to be more severe than in normal individuals. Herpesviruses commonly associated with post-transplant disease include Epstein-Barr virus (EBV), Cytomegalovirus (CMV), Varicella-Zoster virus (VZV) and Herpes-simplex viruses 1 and 2 (HSV 1 and 2) (Roizman 1996). These viruses are discussed in more detail in the next section. Human papillomavirus (HPV) causes large and numerous warts in transplant recipients and is associated with increased incidence of cervical carcinoma in these patients (Bavin *et al* 1992). Hepatitis B virus (HBV) has a high incidence of recurrence in liver transplant recipients following HBV liver disease (O'Grady *et al* 1992). This may result in a spectrum of disease, from asymptomatic infection to fulminant liver failure. Donors are screened for HBV surface antigen (HBsAg) and HBV positive organs rejected for transplantation. Hepatitis C virus (HCV) causes similar, though less severe, disease and therefore HBV and HCV positive donors should not be used for transplantation (Valeri *et al* 1993).

Transplant recipients are at three times greater risk of developing cancers than the general population (Sheil *et al* 1993). The Cincinatti Transplant Tumour Registry (CTTR) monitored worldwide incidence of tumours in 5,753 transplant recipients (Penn 1993). Of 6,123 cancers in this group, the most common type were skin carcinomas (2,261 cases), representing 37% of the total. In areas with strong sunlight, transplant recipients are forty times more likely to develop skin cancers

than the general population (Penn 1994). In Australia, over 50% of transplant recipients have developed cancer by twenty years after surgery and these tumours tend to be more aggressive than in healthy people.

Lymphomas make up 5% of cancers in the general population, whereas they comprise 12-33% of cancers in transplant recipients, with a high preponderance of non-Hodgkins lymphoma (Sheil *et al* 1992, Penn 1994). Over 80% of these lymphomas are of B-cell origin, 12.6% of T-cell origin, and 0.4% of null cell origin. Multiple organ involvement occurs in 52% of transplant recipients with lymphoma, and transplant recipients are at one thousand fold higher risk of cerebral lymphoma (Sheil *et al* 1993a & b). The grafted organ is involved in 23% of cases, which may be misdiagnosed as acute rejection (Penn 1993). Lymphomas occur on average 32 months post-transplant (range 1 to 254 months) with cerebral lymphomas presenting earlier than diffuse lymphomas (average of 2 years and 6 years respectively) (Penn 1994 & 1995).

The greatest risk is from cancers with a viral aetiology, including lymphomas (EBV), cancers of the perineum (HPV), skin cancers (HPV) and Kaposi's sarcoma (HHV8). These tumours tend to occur just a few months or years post-transplant, occur in younger individuals than would normally be expected and have a more aggressive course with earlier dissemination (Sheil *et al* 1992).

1.2 Herpesviridae

The herpesvirus family, *Herpesviridae*, is characterised by virus structure, having a linear double stranded DNA (dsDNA) genome encoding around 160 genes, contained within an icosahedral capsid of 100nm diameter, composed of 162 capsomers. The virion is surrounded by a layer of amorphous tegument enclosed in a host-cell derived lipid envelope containing viral glycoproteins giving the enveloped virion a total diameter of 150-200nm (Roizman 1996). All animals tested to date have at least one associated herpesvirus. The wide distribution and host specificity of herpesviruses suggests close co-evolution with their respective hosts.

Eight human herpesviruses have been isolated so far, namely herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), human cytomegalovirus (HCMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), and human herpesviruses 6 and 7 and Kaposi's sarcoma associated herpesvirus (KSHV or human herpesvirus 8 (HHV8)). The classification and disease association of these viruses is summarised in Table 1-1. The numerous members of *the Herpesviridae* are classified on the basis of their biologic properties, principally the agents host range, length of replicative cycle, site of latent infection and cytopathology (Roizman *et al* 1981). More recently, DNA sequence analysis of Herpesviruses has provided the opportunity to review classification. There are three subfamilies of herpesviruses: Alpha(α)-, Beta(β)- and Gamma(γ)- herpesviridae. Gammaherpesviridae includes two genera; lymphocryptovirus, which includes EBV, and Rhadinovirus. Most Herpesviruses would remain in their original classification, with the exception of Marek's disease virus and HHV6, both of which would be classified as gammaherpesvirinae on the basis of biologic criteria, but by objective criteria, they would be members of the alpha and beta herpesvirinae respectively (Buckmaster *et al* 1988).

Lymphocryptoviruses have only been found in old world primates and humans, each species carrying its own virus which is closely related to the other viruses of the genera. This indicates that a common precursor of EBV-like viruses once infected the hominid species which evolved into humans and the old world primates (Rabin *et al* 1980).

Vernacular and approved names are used interchangeably for human herpesviruses and members of each group. A summary of their classification is shown in Table 1-1.

The known herpesviruses share four common properties:

i. They encode a large number of enzymes involved in nucleic acid metabolism (eg thymidine kinase, dUTPase, ribonucleotide reductase etc.), DNA synthesis (DNA polymerase, helicase, primase), and protein processing (eg protein kinase).The range of enzymes encoded varies between herpesviruses.

ii. Synthesis of viral DNA and capsid assembly occurs in the nucleus and the capsid is enveloped as it transits through the nuclear membrane. The capsid may be re-enveloped as it passes through the cytoplasm.

iii. Production of infectious progeny virus results in destruction of the host cell (lytic replication).

iv. All herpesviruses described are able to remain latent in their natural hosts. During latent infection, the genomes remain as closed circular episomes and a minimal set of viral genes are expressed, allowing the virus to persist in the host.

Table 1-1. Classification of Human Herpesviruses

(adapted from Jawetz *et al* 1989)

	Biologic properties			Examples		
Sub-family	Growth cycle	Cyto-pathology	Latent infections	Name : HHV-	Common name[1]	Disease associated with 1° infection[2]
alpha	Short (~18 hrs)	Cytolytic	Neurons	1 2 3	HSV-1 HSV-2 VZV	Vesicular stomatitis Genital herpes Chicken-pox
beta	Long (~70 hrs)	Cytomegalic	Glands, kidney leukocytes	5 6 7	CMV N/A N/A	IM Exanthum subitum Not known
gamma	Variable	Lympho-proliferative	Lymphoid Endothelium	4 8	EBV KSHV	IM Not known

1. HHV: Human Herpes virus. HSV: Herpes Simplex virus. VZV: Varicella Zoster virus. CMV: Cytomegalovirus. EBV: Epstein-Barr virus. KSHV: Kaposi's Sarcoma associated Herpesvirus.
2. IM: Infectious mononucleosis.

Herpesviruses infect the host cell by fusion with the cell membrane following binding of specific cellular receptors with virus envelope glycoproteins (Roizman 1996). The virus is then uncoated and the DNA becomes associated with the nucleus. Lytic replication begins with expression of "immediately-early" genes, the protein products of which transactivate expression of the "early" set of genes. The immediate early and early gene products encode enzymes or DNA-binding proteins. Viral DNA replication begins by a rolling circle mechanism involving cellular DNA polymerase II and with participation of viral early proteins. "Late" genes are also transcribed which provide structural components. Viral DNA is packaged in preformed nucleocapsids in the nucleus and maturation occurs by budding through the nuclear membrane where the virus gains its envelope, and transportation of the virus particles to the cell surface for release. Herpesvirus replication leads to death of the host cell.

The ability of herpesviruses to establish a life-long latent infection relies on evasion of the host cellular immune system. In the setting of inherited, acquired or iatrogenic immunodeficiency, loss of immune control of herpesvirus infection can lead to fatal disease. The most common cause of herpesvirus infection in the first month post-transplant is CMV, which causes symptoms including malaise, fever and lethargy and can result in interstitial pneumonitis, hepatitis, colitis or retinitis, and may lead to disseminated life-threatening disease from hypotension and respiratory failure (Peterson *et al* 1980). Patients most vulnerable to CMV disease post-transplant are CMV seronegative transplant recipients receiving an organ from a CMV positive donor (Paya *et al* 1993b). Therefore it is recommended that donors and recipients are CMV matched (Nicholson *et al* 1994) and transplant centres employ prophylaxis to prevent CMV disease using ganciclovir (Meriden *et al* 1992) and immune globulin (Snydman *et al* 1987).

Herpes simplex virus 1 and 2 generally cause disease in transplant recipients through reactivation of virus from their site of latency in trigeminal ganglia. HSV disease is generally seen between the first week and first month after transplantation and results in marked localised ulceration or widespread dissemination, leading to local or disseminated skin rash or cold sores, oesophagitis, pneumonitis, hepatitis or encephalitis. Ophthalmic infection can result from patients rubbing their eyes.

Treatment for HSV disease is through the routine prophylactic use of the inhibitor of DNA replication acyclovir (ACV) in the early post-operative period (Saral *et al* 1983).

VZV also generally causes disease through reactivation from dorsal root ganglia, leading to shingles early post-transplant. Patients may have extensive vesicles involving the epidermis and dermis which are often haemorrhagic or necrotic. Disseminated disease has a high mortality, and patients who undergo primary infection with VZV post-transplant suffer from an aggressive chicken pox with a high risk of mortality. VZV disease can be prevented through use of live attenuated vaccine prior to transplantation or treated with ACV (Kitai *et al* 1993).

Transplant recipients are also at risk of developing HHV-8 associated Kaposi's sarcoma (Sheil *et al* 1993a). EBV is associated with post-transplant lymphoproliferative disease (PTLD), which is discussed further in section 1.3.11.8.

1.3 Epstein-Barr virus (EBV)

1.3.1 Introduction

In 1958, Dennis Burkitt, a British surgeon working in Uganda, described a malignancy of the jaw occurring in African children which is now known as Burkitt's lymphoma (BL), a tumour of B-cell origin (Burkitt 1958). The distinct geographical restriction of BL in equatorial Africa suggested an infectious origin of the disease and because of its coincidence with the distribution of malaria it was suggested that mosquitoes provided the vector for the agent (Burkitt 1962). Cell lines were subsequently established *in vitro* from BL tumour material and electron microscopy of these cells identified herpesvirus particles which were later shown to be unique and named Epstein-Barr virus (EBV) (Epstein & Barr 1964, Epstein, Achong and Barr 1964). BL is discussed in detail in section 1.3.11.1.

EBV infects B-, T- and epithelial cells *in vitro* and *in vivo* and like the other herpesviruses maintains a lifelong infection, termed latency, which allows persistence of the virus in infected cells through repression of lytic replication. EBV is the causative agent of infectious mononucleosis (IM) (Henle *et al* 1968) and

aetiologically associated with a number of epithelial and lymphoid tumours in the immunocompetent and immunosuppressed host (Rickinson & Kieff 1996).

1.3.2 EBV genome

EBV is a large (around 120nm) gammaherpesvirus with a linear dsDNA genome of 172 kilobase-pairs. EBV was the first herpesvirus whose genome was completely sequenced (Baer *et al* 1984). The laboratory prototype strain of EBV is B95-8, which was derived from marmoset B-cells infected with EBV from throat washings from a patient with infectious mononucleosis (Miller *et al* 1973). The EBV genome has a guanosine-cytosine (G-C) content of 60%, and has a characteristic arrangement of repeat regions. These are a reiterated 0.5kbp terminal repeat (TR) region of variable length and a reiterated 3kbp internal repeat (IR1) which divides the genome into short and long largely unique sequence domains (US and UL). Within the unique long (UL) region are three specific repeats IR2, 3 and 4. Covalent linking of the TR regions forms a circular episome in the nucleus of a latently infected cell (Nonoyama & Pagano 1972. Lindahl *et al* 1976). An individual cell infected with EBV contains multiple identical (clonal) copies of the EBV episome, replicated by cellular DNA polymerase (Sugden *et al* 1979).

The EBV genome sequence was derived from a Bam H1 fragment cloned library, and therefore genome organisation of open reading frames (ORFs), genes or transcription sites are named with reference to Bam H1 fragments. EBV genes have a four-letter and number acronym based on the restriction fragment in which transcription begins; for example BZLF1 refers to the first (1) leftward (L) ORF (F) of the Bam H1 (B) Z fragment (Z) of the EBV genome (Baer *et al* 1984). The organization of the EBV genome is represented in Figure 1-1.

1.3.3 EBV Types

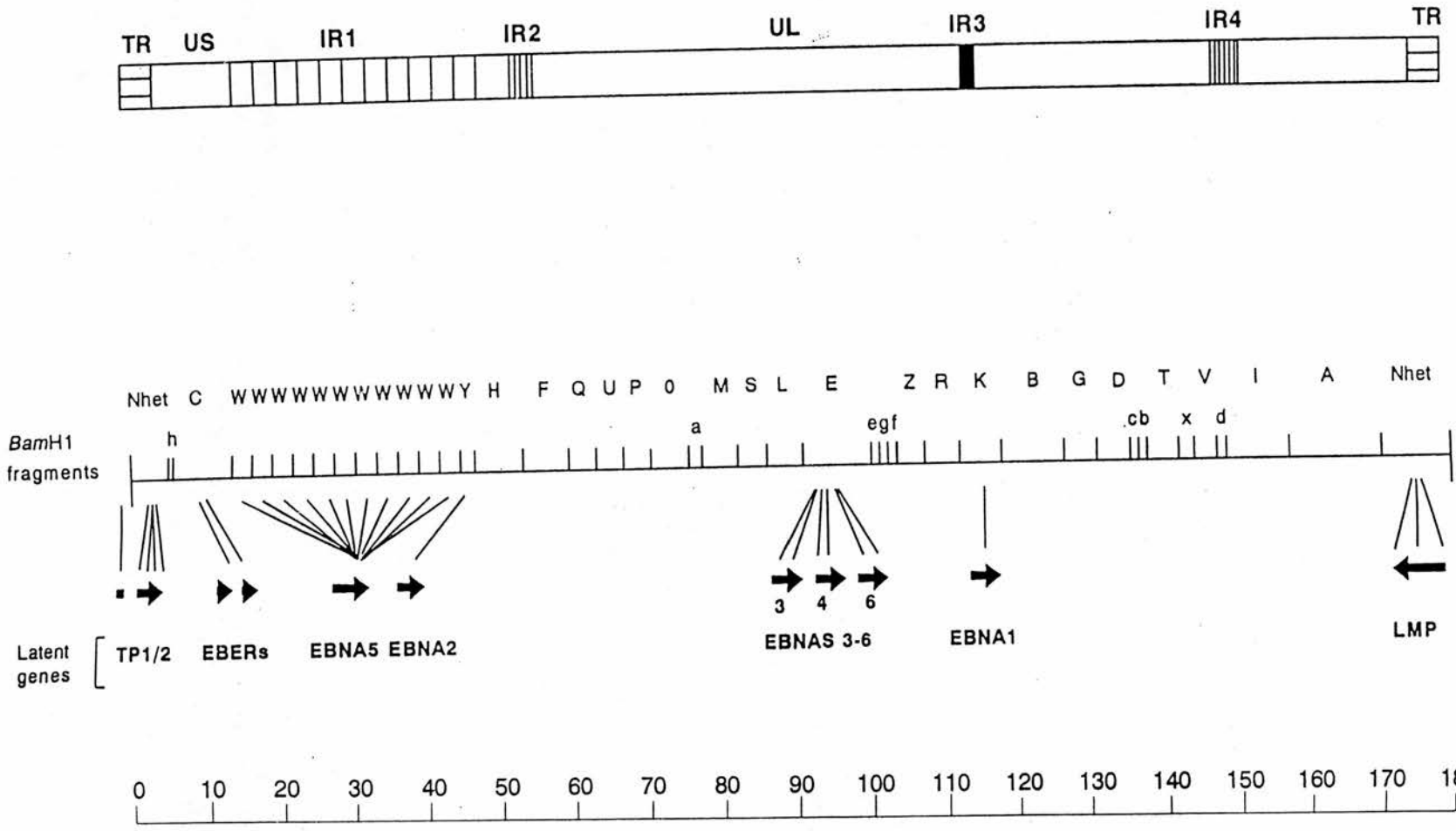
EBV has two prototype virus types; A and B (or type 1 and 2) based on genome sequence differences (Dambaugh *et al* 1980). The types differ in the sequence of genes encoding Epstein-Barr virus encoded nuclear antigen 2 (EBNA2) EBNA 3a, b and c (Rowe *et al* 1989, Sculley *et al* 1989, Sample *et al* 1990) and the

EBV encoded small RNAs (EBERs) 1 and 2 (Arrand, Young and Tugwood 1989). The role of these genes is described in Section 1.3.7. There is a geographical and ethnic difference in the frequency of the two types of EBV although both types exist worldwide (Zimber *et al* 1986, Sixby *et al* 1989). Type 2 EBV is more common in African populations and type 1 more common in European or US populations (Zimber *et al* 1986). Although type 2 EBV is less efficient at transforming B-lymphocytes *in vitro* (Rickinson *et al* 1987), the different EBV types are not preferentially associated with specific diseases (Lung *et al* 1988).

Figure 1-1. Diagram of the layout of the linear EBV genome and BamH1 restriction enzyme map

Upper panel: A schematic diagram of the linear EBV genome.
IR: internal repeat sequence. TR: Terminal repeat sequence. US: Unique short sequence. UL: Unique long sequence.

Lower panel: A schematic diagram of the linear EBV genome indicating the BamH1 restriction enzyme fragments obtained by enzymatic digestion of the prototype EBV strain, B95-8. Acronyms denote open reading frames of the known latent genes, the exons of which are indicated on the diagram.
EBER: EBV RNA. EBNA: EBV nuclear antigen complex. LMP: Latent membrane protein.


TR
US
IR1
IR2
UL
IR3
IR4
TR
Nhet C WWWWWWWWWWWY H F Q U P O M S L E Z R K B G D T V I A Nhet
BamH1 fragments
h
a
egf
cb x d
3 4 6
Latent genes
TP1/2
EBERs
EBNA5 EBNA2
EBNAS 3-6
EBNA1
LMP
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180

1.3.4 EBV Isolates

EBV isolates can be characterised on the basis of the number of sequence repeats within repeat regions present in the viral genome. In practice, the isolates are identified by genotyping or EBNO-typing. Genotyping is carried out by restriction fragment length polymorphism (Katz *et al* 1988) or by PCR analysis (Sixby *et al* 1989). EBNO-typing is performed by generating lymphoblastoid cell lines (LCLs) and analysis of EBNA protein size polymorphism by immunoblotting (Gratama *et al* 1990, 1994). Normal healthy EBV seropositive donors carry a single EBV isolate which is consistently detectable in both blood and throat washings (Yao *et al* 1991). Immunosuppressed patients may carry multiple EBV isolates (Miller *et al* 1987, Yao *et al* 1996a and b).

1.3.5 EBV encoded transcripts and proteins

The large genome of EBV (172 kbp) contains 84 major unique ORFs (Baer *et al* 1984). EBV is able to express different combinations of genes in latency and lytic infection through differential promoter usage. Viral messenger RNA (mRNA) transcription is mediated through host cell RNA Polymerase II since herpesviruses do not encode their own RNA polymerase.

1.3.6 Models of EBV Latency

EBV expresses different combinations of latent genes, which are characteristic of particular cell types or disease states. These combinations have been broadly classified in three groups termed Latency I, II and III, described in detail below (Rowe *et al* 1992). EBERs and Bam H1 A and EBNA1 transcripts are detected in all forms of latent gene expression.

Differential expression of the EBV latent genes is through use of alternative promoters. Transcription of the EBNA gene complex occurs from a single transcriptional unit spanning 100 kilobases of the EBV genome. One of two promoters, Cp or Wp, drive transcription of the EBNAs, and alternative splicing

leads to synthesis of mature EBNA transcripts (Kieff 1996). Following infection of B-lymphocytes *in vitro*, EBNAs are initially transcribed from Wp and promoter usage subsequently switches to Cp (Woisetschlager *et al* 1990). Cp or Wp operate mutually exclusively in individual EBV infected cell lines *in vitro* (Woisetschlager *et al* 1989). During Latency I *in vitro* and *in vivo*, transcription from Cp or Wp is repressed through methylation and EBNA1 is transcribed from a distinct promoter, Qp (Nonkwelo *et al* 1996, Paulson & Speck 1999). Cp and Wp are extensively methylated in EBV episomes from normal healthy EBV carriers and in tumours and cell lines displaying restricted EBV latency, whereas Qp is consistently hypomethylated (Schaefer, Strominger and Speck 1997, Paulsson & Speck 1999). Demethylation of Cp/Wp by 5-azacytidine, a methyltransferase inhibitor, leads to activation of EBNA gene transcription from Cp/ Wp (Masucci *et al* 1989, Altiok *et al* 1992, Jansson, Massuci and Rymo 1992, Robertson *et al* 1995). EBNA1 positively regulates Cp and Wp promoter activity whereas it negatively regulates transcription from Qp (Puglielli, Woisetschlager & Speck 1996, Sample, Henson and Sample 1992, Schaefer, Strominger and Speck 1997). This is reflected in the variable detection of Qp initiated EBNA1 transcripts in healthy EBV carriers since in quiescent memory B-cells, there may be sufficient EBNA1 to repress Qp activity.

1.3.6.1 Latency I

This form of latent gene expression is characterised by transcription of EBNA1 from the Qp promoter with no transcription of the other EBNAs from the silent Cp or Wp promoters (Nonkwelo *et al* 1996, Schaefer *et al* 1995). The LMPs are not transcribed (Brooks *et al* 1995, Chen *et al* 1995). This pattern of EBV gene expression is detected in Burkitt's lymphoma cells in biopsy material and early passage BL lines (Rowe *et al* 1986, 1987).

1.3.6.2 Latency II

Latency II is characterised by expression of EBNA1 from Qp with no transcriptional activity from Cp or Wp. In addition, one or more of the LMP promoters are active, resulting in expression of LMP1, LMP2a and/or LMP2b (Kerr *et al* 1992). This form of latent gene expression is detected in Reed-Sternberg cells in

Hodgkin's disease (HD) and in the malignant epithelial cells of nasopharyngeal carcinoma (NPC) (Brooks *et al* 1992, Deacon *et al* 1993).

1.3.6.3 Latency III

This form of gene expression is detected in latently infected cells in LCLs and in peripheral blood mononuclear cells (PBMs) in early infectious mononucleosis (IM) and PTLD tumour cells (Thomas *et al* 1990a, Rickinson and Keiff 1996). The Cp or Wp promoters are active, resulting in expression of all the EBNAs and all three LMPs.

In vitro passage of BL cell lines frequently leads to drift from latency I to latency III pattern of EBV gene expression, with a concomitant loss of viral gene methylation (Minarovits *et al* 1991). It appears that EBV utilizes the host cell methylation system to regulate expression of antigenic latent proteins, allowing the virus to evade the host immune system and establish a lifelong latent infection.

1.3.7 Latent cycle transcripts

In vitro infection by EBV of mature, surface immunoglobulin (sIg) positive B-lymphocytes results in cellular activation and the establishment of an immortalised B-LCL, blocked in the lymphoblastoid stage of B-lymphocyte differentiation (Thorley-Lawson & Mann 1985). The majority of LCL cells contain multiple copies of the non-replicating episomal form of the EBV genome and only a small proportion of cells contain replicating virus (Lindahl *et al* 1976). LCLs provide a model for Latency III since they express all nine viral latent antigens (See Table 1-2). This includes six EBNAs: -1, -2, -3a, 3b and 3c and Leader protein (-LP)) and three LMPs: 1, 2a and 2b (Reviewed in Kieff 1996). Abundantly expressed EBERs 1 and 2 are transcribed but not translated (Clemens 1993) and the transcript from the Bam H1A reading frame is translated into protein(s) although its nature and function are unclear (Brooks *et al* 1995, Fries *et al* 1997). Promoter activity and transcribed genes in latency I and III is shown in Figure 1-2.

Deletion analysis has shown that EBNAs -1, -2, -3a and 3c and LP and LMP1 are essential for host cell growth immortalisation (Reviewed in Rickinson & Keiff

1996). The function of these proteins is described in this chapter (section 1.3.6). There is an alternative nomenclature for the EBV latent antigens; EBNA-LP is also known as EBNA-5 and EBNAs 3a, 3b and 3c are known as EBNA-3, -4 and -6 respectively. LMP1, 2a and 2b are known as LMP, terminal protein (TP) 1 and 2 respectively.

Individual mRNAs are generated by differential splicing, poly (A) site selection and alternative promoter usage (Speck & Strominger 1985, Woisetschlager *et al* 1989, Sample *et al* 1991). The role and function of the individual EBNA proteins are described below.

Table 1-2 EBV latent transcripts

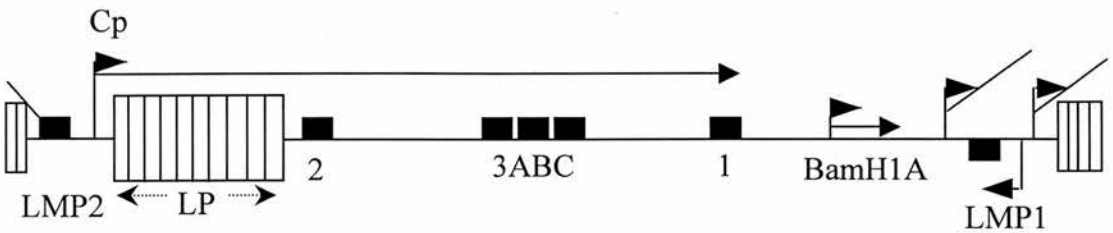
(Adapted from Johannessen and Crawford 1999)

Protein	ORF	Molecular weight (kDa)	Cellular site	Required for immortalisation
EBNA 1	BKRF1	65-97	Nucleus	Yes
EBNA 2	BYRF1	75-105	Nucleus	Yes
EBNA 3a	BLRF3/ BERF1	130-195	Nucleus	Yes
EBNA 3b	BERF2a/ b	145-160	Nucleus	No
EBNA 3c	BERF3/ 4	130-195	Nucleus	Yes
EBNA LP	BWRF1	20-130	Nucleus	Yes
LMP1	BNLF1	58-63	Membrane	Yes
LMP2a	BARF1/BNRF1	54	Membrane	No
LMP2b	BNRF1	40	Membrane	No
EBER 1/2	BCRF1	-	Nucleus/ cytoplasm	No
Bam H1 A	BARF0	-	cytoplasm	No

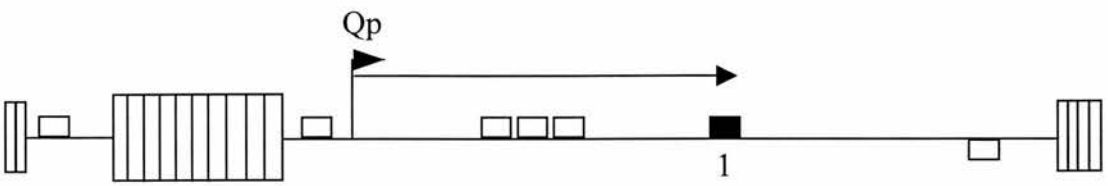
Figure 1-2. EBV transcription in latency

(Adapted from Farrell and Sinclair 1994)

Latent promoter activity in EBV immortalised B-cell lines (LCL): Latency III



Latent promoter activity in Burkitt's lymphoma cells



Diagrams show the gene layout in immortalised cells compared to Burkitt's lymphoma. EBV promoter activity and expressed latent genes are shown. Arrows show active promoters and the direction of transcription. The coding segments of EBNA1, 2,3a, 3b, 3c, LMP1, 2a and 2b are illustrated as filled boxes in the upper diagram. EBNA-LP (dashed arrows) is composed of many small exons crossing the major internal repeat.

1.3.7.1 EBERs

EBERs 1 and 2 are the most abundantly expressed EBV transcripts in latently infected B-cells (Rymo 1979, Arrand and Rymo 1982). These small (167 and 172 nucleotides respectively), non-translated RNAs (Clemens 1993) are transcribed from hypomethylated transcription units by host cell RNA polymerase III (Clarke *et al* 1992, Minarovits *et al* 1992). EBERs exist in the nucleus as ribonucleoprotein particles complexed with the ribosomal protein L22 (Toczyski *et al* 1994). The L22 gene is a target for chromosomal translocation in certain patients with myeloid leukaemia and EBERs may therefore have a role in stabilizing cellular transformation. EBERs are also detected in the cytoplasm where they bind p68, an interferon-induced dsRNA dependent protein-kinase, which may protect EBV protein synthesis from interferon induced inhibition (Schwemmle *et al* 1992, Clarke *et al* 1991 & 1992). EBERs are not essential for B-cell immortalisation *in vitro* (Swamminathan *et al* 1991). However studies in the BL cell line Akata in which EBV negative clones were transfected with EBERs constructs indicated that they contribute to apoptosis resistance and the malignant phenotype (Komano *et al* 1999).

1.3.7.2 EBNA-1

EBNA1 is the only EBV encoded protein to be expressed in all EBV associated malignancies (Klein 1989, 1994). It is a nuclear antigen encoded by the BKRF1 gene containing a variable length internal repeat (IR3) encoding the amino acids glycine and alanine (gly-ala repeat) flanked by arginine rich sequences (Henessy & Keiff 1983). Other important domains of EBNA1 include a DNA looping region, nuclear localization signal, DNA binding and dimerisation domain and a C-terminal tail (Mackey *et al* 1995, Bochkarev *et al* 1996, Kieff 1996).

EBNA1 is essential for maintenance of the viral episome through binding to the EBV origin of replication (*Ori*P) (Middleton & Sugden 1994) and is also responsible for partitioning of progeny EBV genomes to daughter cells in mitosis through associating with the host cell chromosomes (Adams 1987, Yates & Guan 1991, Davenport & Pagano 1999). Although the EBV origin of replication (*Ori*P) is independently capable of recruiting human cellular replication factors and initiating

genome replication, EBNA1 is required to ensure continued replication and transcription (Polvino-Bodnar & Schaeffer 1992, Aiyar *et al* 1998). *Ori*P is composed of two clusters of EBNA1-binding sites (Rawlins et al 1985, Reisman *et al* 1985). EBNA1 interacts with a human single stranded DNA binding protein, (hSSB, also called RPA), which may facilitate recruitment of other host replication proteins to replicate the EBV genome (Zhang *et al* 1998).

*Ori*P is also a transcriptional enhancer through which EBNA1 supports the activity of at least two EBV promoters: Cp and the LMP1 promoter (Puglielli *et al* 1996 & 1997, Gahn & Sugden 1995, Reisman & Sugden 1986, Sugden & Warren 1989). Unless Cp/ Wp are inactivated through methylation, EBNA1 activates transcription from these promoters and represses Qp activity (Paulsson & Speck 1999).

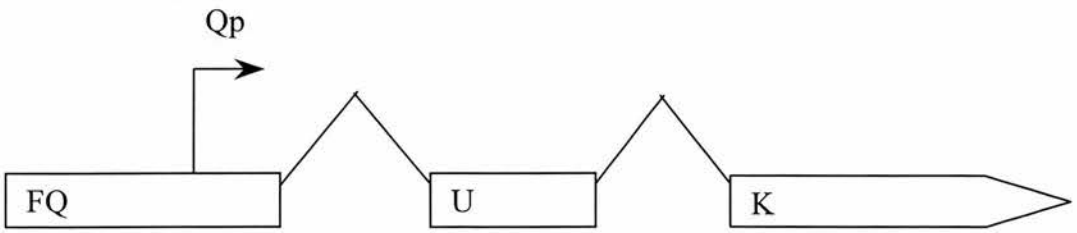
Although the principal role of EBNA1 appears to be in maintaining the EBV genome, the fact that it is expressed in all EBV-associated tumours suggests a potential role in tumorigenesis. Wilson *et al* (1996) demonstrated that EBNA1 transgenic mice develop lymphomas at increased frequency. It has been shown that EBNA1 upregulates expression of recombinase activating genes (RAG) which are involved in regulating immunoglobulin gene VDG rearrangement (Srinivas & Sixby 1995). Aberrant VDJ recombination in EBV infected B-cells could potentially facilitate MYC translocation to immunoglobulin loci, the characteristic translocation in Burkitt's lymphoma.

Differentially expressed transcripts of EBNA1 are produced by alternative promoter usage. This occurs from the Cp or Wp promoter in LCLs or the Qp promoter in BL cell lines (Sample *et al* 1991). In cells undergoing lytic replication, EBNA1 is the sole EBNA complex gene expressed, in this case transcribed through another EBNA1 promoter, Fp (Ernberg *et al* 1976, Weigel and Miller 1983, Weigel *et al* 1985). EBNA1 regulates its own expression from Qp through interaction with a binding site within the Bam H1 Q fragment (Rawlins *et al* 1985, Sample *et al* 1992). Zetterberg *et al* (1999) performed RNAse protection assays to quantify the levels of EBNA1 transcripts in B-lymphoid cells in EBV latency and under induction of lytic replication. During lytic replication low levels of FpQ/ U/ K spliced EBNA1 were

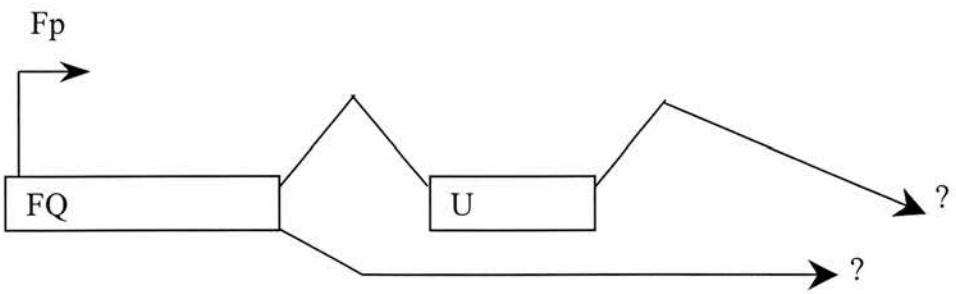
present in all cells. In addition high levels of transcripts initiating in Fp were detected, some of which contain the U exon, but which did not contain the EBNA1 encoding K exon, and map to unknown 3' sequences (see Figure 1-3) (Schaefer *et al* 1995).

Figure 1-3. Promoter activity in EBNA1 transcription

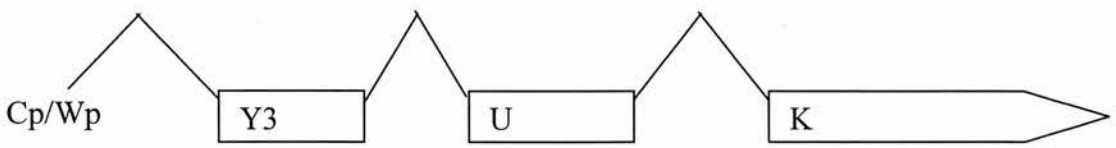
Latent transcription of EBNA1



EBNA1 Promoter activity in lytic replication



EBNA1 transcription during Latency III



Figures represent different patterns of promoter usage and exon transcription in alternative programs of EBV latency and lytic replication, as described in the text (Section 1.3.7.2). Transcribed exons are represented by boxes with linking diagonal lines representing slicing across introns. The EBNA1 coding exon (K) is shown as the pointed box. Promoters are indicated (Qp, Fp, Cp/Wp). Transcripts initiating from Fp splice to the U exon, or bypass it, but the downstream splice sites are not known, indicated by the arrows leading to question marks.

Despite the fact that EBNA1 is antigenic and raises an antibody response (Dillner *et al* 1984), cytotoxic T-cell (CTL) responses are not usually detected against EBNA1 (Rickinson & Moss 1997). This is due to inhibition of the ubiquitin/proteosome dependent degradation pathway by the gly-ala repeat sequence of EBNA1 which prevents the production of peptides for major histocompatability complex (MHC)-1 restricted presentation (Levitskaya *et al* 1995, 1997). CTL responses can be raised against EBNA1 if antigen-presenting cells process the protein exogenously *in vitro,* and this pathway may be responsible for the occasional detection of CTL reactivities against EBNA1 epitopes (Blake *et al* 1997). EBNA1 homologues are found in other primate lymphocryptoviruses yet despite a high degree of conservation and the presence of gly-ala repeats rhesus monkey and baboon isolates do not inhibit antigen processing and presentation (Blake *et al* 1999).

1.3.7.3 EBNA-2

EBNA2 is a 75-105kDa protein, which, with EBNA LP, is one of the first EBV genes expressed following *in vitro* infection of B-lymphocytes. EBNA2 is essential for B-cell immortalisation and reintroduction of wild-type EBNA2 into B-lymphocytes infected with P3HR1, a strain of EBV in which EBNA2 is deleted, restores immortalising activity (Hammerschmidt & Sugden 1989, Cohen *et al* 1989). EBNA2 has a strong transactivating domain which does not bind DNA directly but can interact with multiple constituents of the RNA polymerase II complex (Tong *et al* 1995 a, b & c). The function of EBNA2 mimics signalling through the Notch pathway (Strobl *et al* 2000, Hsieh *et al* 1997). EBNA2 upregulates expression of LMP1 (Abbott *et al* 1990, Wang *et al* 1990) and LMP2a and b (Zimber-Strobl *et al* 1991 & 1993) and mediates promoter switching from Wp to Cp following initial transcription from Wp immediately after B-cell infection (Woisetschlager *et al* 1991, Jin & Speck 1992). Thus EBNA2 has an autoregulatory function through upregulating its own promoter (Cp) (Puglielli *et al* 1997). EBNA2 binding to enhancer elements upstream of these promoters is mediated by recombinant signal binding protein (RBP) Jκ, also known as CBF1 (C promoter binding factor 1) (Tsang *et al* 1991, Zimber-Strobl *et al* 1993, Henkel *et al* 1994, Krauer *et al* 1999). It can

also be recruited to promoters by cellular factors including PU1 or transcription factors of the POU family (Pit-Oct-Unc) (Johannsen *et al* 1995). RBP is a transcriptional repressor which regulates cell differentiation, and in LCLs it is sequestered from the cytoplasm to the nucleus where it is bound in chromatin by EBNA2 and EBNAs 3a, b and c (Krauer *et al* 1999). Around half of the cellular RBP is associated with EBNAs in LCLs and the majority of EBNA2 in an LCL is associated with RBP (Johannsen *et al* 1996). Sequestering of RBP by EBNAs may block further differentiation in infected cells (Hsieh *et al* 1997).

EBNA2 also transactivates a number of cellular genes, including CD21 (Cordier *et al* 1990), CD23 (Wang *et al* 1991), c-fgr (Knutson 1990) and directly transactivates the proto-oncogene *c-myc* (Kaiser *et al* 1999). EBNA2 also acts as a suppressor, efficiently suppressing IgM expression (Jochner *et al* 1996). If *c-myc* is overexpressed in an inducible system, B-cell proliferation can continue in the absence of EBNA2 and LMP1 (Polack *et al* 1996). This indicates a mechanism for continued proliferation of cells containing a MYC/Ig translocation as seen in Burkitt's lymphoma, independently of EBNA2 and LMP1 expression. In cooperation with EBNA-LP, EBNA2 can induce cyclin D2 and cyclin-dependent kinase 4 (cdk4) in resting B-lymphocytes causing cell cycle progression from G0 to G1 (Sinclair *et al* 1994). Thus EBNA2 plays a vital role in the process B-cell immortalisation by EBV through inducing proliferation and blocking differentiation by a variety of mechanisms.

1.3.7.4 EBNA 3a, 3b and 3c

EBNAs 3a, 3b and 3c derive from three tandemly located genes within the Bam H1 E fragment, each transcript consisting of a shared leader sequence followed by a short and long exon. EBNA3a is encoded by BLRF3 and BERF1, EBNA3b by BERF2a and BERF2b and EBNA3c by BERF3 and BERF4. EBNA3a and 3c vary in size from 130 to 195kDa and EBNA3c from 145- 160kDa (Ricksten *et al* 1988).

EBNA3a and 3c are essential for B-lymphocyte immortalisation whereas EBNA3b is not (Tomkinson & Kieff 1992). EBNA3b up-regulates the cytoskeletal

protein vimentin and the activation antigen CD40, but down regulates the BL associated antigen CD77 when it is stably expressed in the BL cell line DG75 (Silins & Sculley 1994).

EBNA3c is an immortalising oncoprotein with similar properties to the pRb inactivating proteins of other DNA tumour viruses (for example adenovirus E1A and papillomavirus E7), driving the cell cycle through G1 under conditions which would normally signal growth arrest (Parker *et al* 1996, 2000). It is a DNA binding protein containing a basic leucine zipper motif, characteristic of c-fos and c-jun (Vinson *et al* 1989, Allday *et al* 1993) and can cooperate with (Ha)-ras in co-transfection assays to immortalise and transform rat embryo fibroblasts (Parker *et al* 1996). EBNA3a and 3c interact with RBP Jκ and repress the effects of EBNA2 mediated transcription through destabilizing RBP Jκ binding to DNA (Waltzer *et al* 1996, Hsieh *et al* 1997a & b). Thus EBNA3a and 3c inhibit the transactivation of LMP1 and 2 by EBNA2 which may be important in preventing toxicity through over expression of LMP1 (Le Roux *et al* 1994, Marshall and Sample 1995, Robertson *et al* 1995). EBNA3a and 3c directly repress transcription *in vitro* when tethered to a promoter via the DNA binding domain of yeast Gal4 protein (Bain *et al* 1996, Waltzer *et al* 1996, Bourillot *et al* 1998). EBNA3c inhibits Cp transcription through Jκ (Radkov *et al* 1997, Bain *et al* 1999), providing autoregulation of EBNA gene expression. EBNA3c also inhibits transcription through promoting nucleosome formation by modulating histone deacetylase function (Cotter & Robertson 2000, Radkov 1999). Since EBNA2 can transactivate via RBP Jκ or PU1, inhibition of RBP Jκ mediated transcription by EBNA3 may provide differential regulation of viral and cellular genes including upregulation of CD21, CD23 and LMP1 (Wang *et al* 1990, Allday & Farrell 1994).

1.3.7.5 EBNA-LP

EBNA-LP (leader protein) is so called because its coding sequence precedes the other EBNA coding sequences in all EBNA transcripts. The size of the EBNA-LP protein varies between 20 to 130kDa protein due to alternative splicing of

multiple repeat exons in the Bam H1 W exon which link onto the short coding sequences in the Bam H1 Y fragment (Dillner *et al* 1986, Dillner & Kallin 1988).

EBNA-LP affects cell growth, providing a role in enhancing B-lymphocyte immortalisation by EBV. The efficiency of EBV in producing LCLs following infection of primary human B-lymphocytes *in vitro* is reduced by 90% if EBNA-LP is deleted. LCLs produced in the absence of EBNA-LP require a feeder layer for growth, and are not long lived (Mannick *et al* 1991, Allan *et al* 1992). EBNA-LP localises to areas in the cell nucleus called ND10, (also known as PML (pro-myelocytic leukaemia) bodies and associates with the interferon induced chaperone protein heat shock protein 70 (hsp70) (Petti *et al* 1990, Mannick *et al* 1995, Szekeley *et al* 1996, Kitay *et al* 1996). *In vitro*, EBNA-LP weakly binds p53 and pRb (Szekeley *et al* 1993) but it does not affect their role in transcriptional regulation (Inman & Farell 1995). EBNA-LP has a regulatory role in cellular immortalisation, acting in cooperation with EBNA2 in mediating activation of the LMP1 promoter, and of the LMP1 and LMP2b bi-directional transcriptional regulatory element. The stimulatory activity of EBNA-LP resides in an amino terminal sixty-six amino acid repeat domain and c-terminal regulatory domains (Harada & Kieff 1997).

1.3.7.6 LMP1

LMP1 is encoded by the BNLF1 open reading frame, generated from a bi-directional promoter (LMP1p), which also transcribes LMP2b (Laux *et al* 1989). The promoter has two upstream enhancer elements, one of which is EBNA1-dependent (Gahn & Sugden 1995), and the other EBNA2 dependent (Abbott *et al* 1990).

LMP1 is a 386 amino acid protein consisting of six hydrophobic membrane spanning domains which mediate oligomerisation, required for its function (Floettmann & Rowe 1997, Gires *et al* 1997). Immunofluorescence demonstrates aggregates of LMP1 in the cytoplasmic membrane which occur through association of LMP1 with the cytoskeletal protein vimentin (Liebowitz *et al* 1987, Mann *et al* 1985, Liebowitz *et al* 1986, Mann & Thorley-Lawson 1987). Three loops of the

protein are exposed on the outer surface of the cytoplasmic membrane and both termini are cytoplasmic (Liebowitz *et al* 1986).

LMP1 is the major oncogene of EBV and is essential for immortalisation of B-lymphocytes (Kaye *et al* 1993). Rodent fibroblasts transfected with LMP1 are morphologically transformed and are tumorigenic in nude mice (Wang *et al* 1985, Baichwal & Sugden 1988). Transgenic mice with constitutively active LMP1 in epidermis have hyperplasia (Wilson *et al* 1990, Kulchwit *et al* 1998). Transient expression of LMP1 in primary B-lymphocytes *in vitro* leads to cellular enlargement, upregulation of B-lymphocyte activation markers and adhesion molecules and transient induction of DNA synthesis (Peng & Lundgren 1992). LMP1 induces prolonged cell viability although in the absence of EBNA2 is not capable of sustaining B-lymphocyte proliferation (Zimber-Strobl *et al* 1996). LMP1 has also recently been shown to be secreted by LCLs *in vitro* and have direct immunosuppressive effect through suppression of T-lymphocyte activation (Dukers *et al* 2000).

LMP1 forms aggregates in the cell membrane which mimic a constitutively active CD40 cellular receptor and induces extrafollicular B-lymphocyte differentiation, but unlike CD40, LMP1 blocks the germinal centre phenotype (Martin & Sugden 1991, Gires *et al* 1997, Uchida *et al* 1999).

The C-terminus of the protein is essential for *in vitro* immortalisation of B-lymphocytes (Kaye *et al* 1995, Kilger *et al* 1998). The C-terminus contains two functional domains, termed C-terminal activating regions (CTAR) 1 and 2 (Huen *et al* 1995). CTAR1 mediates direct interaction between LMP1 and tumour necrosis factor receptor (TNFR) associated factor (TRAF) and CTAR2 interacts with TNFR-associated death domain (TRADD), leading to activation of the cellular transcription factor NFκB (Hammerskjold & Simurda 1992, Laherty *et al* 1992, Mitchell & Sugden 1995). Thus LMP1 expoits the signal transduction pathways activated by TNF leading to upregulation of NFkB responsive genes (Mosialos *et al* 1995). LMP1 upregulates the anti-apoptotic genes bcl-2 (Henderson *et al* 1991, Rowe et al 1994) and the zinc-finger protein A20 (Laherty *et al* 1992, Fries *et al* 1999, 1996). A20 also modulates transcriptional activation by LMP1 by blocking NFkB activation

(Eliopoulos *et al* 1999). LMP1 induces activity of AP1, a dimer of Jun-Jun or Jun-Fos family of proto-oncogenes, specifically via the c-jun N-terminal kinase 1 (JNK1) pathway (Gires *et al* 1999, Eliopoulos & Young 1998, Kieser *et al* 1997 & 1999). LMP1 also activates the JAK/ STAT pathway which is activated in uninfected cells by almost all cytokines (Gires *et al* 1999, Briscoe *et al* 1996).

LMP1 has diverse and profound effects on infected cell growth, promoting cellular activation and differentiation through numerous pathways whilst inhibiting apoptosis (Izumi *et al* 1999).

1.3.7.7 LMP 2a and 2b

LMP2a and 2b are highly hydrophobic proteins of 54kDa and 40kDa respectively. They are encoded by sequences situated at either end of the EBV genome, in the BARF1/BNRF1 open reading frames and can only be transcribed when the genome is circularised to form an episome (Laux *et al* 1988). The transcripts are encoded across the fused TR of the EBV episome, initiated from separate promoters. The LMP2b promoter is bidirectional, and also initiates transcription of LMP1 (Laux *et al* 1989, Sample *et al* 1989). The mRNAs encoding each protein have a unique 5' exon and eight exons common to both transcripts. Both proteins have twelve transmembrane domains which aggregate in the plasma membrane (Longnecker & Keiff 1990) but LMP2a has an amino terminal cytoplasmic domain whereas LMP2b does not. Neither LMP2a nor 2b are essential for B-lymphocyte immortalisation although they are both expressed in viral latency and in lytic replication (Longnecker *et al* 1992, 1993 a&b. Speck *et al* 1999). They are believed to play a role in facilitating the immortalisation process (Brielmeier *et al* 1996).

LMP2a forms patches in the plasma membrane of B-lymphocytes and is phosphorylated on the tyrosines and serines in its cytoplasmic domain by members of the src family of mitogen activated protein kinases (MAPK) (Fruehling & Longnecker 1997, Panoussis & Rowe 1997, Fruehling *et al* 1998). MAPK directly or indirectly participate in growth factor receptor regulation through phosphorylation of

receptors and associated molecules (David *et al* 1995, Morrison *et al* 1996). When stably expressed in the EBV negative BL cell line BJAB, LMP2a significantly inhibits elevation of intracellular calcium ion levels which normally follows surface immunoglobulin (sIg) receptor or MHCII cross-linking (Burkhardt *et al* 1991, Miller *et al* 1994). In epithelial cells, LMP2a phosphorylation is triggered by cell adhesion in *in vitro* assays through csk, a negative regulator of src (Scholle *et al* 1999). An ITAM motif on the amino-terminus is required for LMP2a function, thus LMP2a but not 2b inhibits src activation (Miller *et al* 1993). Thus LMP2a prevents MAPK activity, leading to inhibition of B-lymphocyte receptor mediated signal transduction (Freuhling & Longnecker 1997). Following surface Ig (sIg) cross-linking of LCLs *in vitro*, LMP2a blocks induction of BZLF1 expression leading to suppression of viral replication (Miller *et al* 1994, 1995) inhibiting EBV reactivation from latency to lytic replication. LMP2a probably has a similar role *in vivo* (Babcock *et al* 1998).

In transgenic mice constitutively expressing LMP2a, B-lymphocytes expressing LMP2a survive despite the absence of normal B-lymphocyte receptor signals. This indicates that LMP2a may enable cellular longevity by minimising normal B-lymphocyte receptor signalling (Caldwell *et al* 1998). LMP2b may have a role in regulating the activity of LMP2a by modulating the spacing between individual LMP2a molecules in aggregates in the plasma membrane (Longnecker & Miller 1996).

1.3.8 EBV Lytic Replication

EBV lytic replication results in release of progeny virus and host cell death. The majority of cells in an LCL contain non-replicating episomal EBV DNA and only 0.1-5% of cells undergo spontaneous switching to lytic replication with release of infectious virus (Henle & Henle 1966, Zur Hausen *et al* 1978). Lytic replication can be further induced by sIg crosslinking, phorbol ester or sodium butyrate treatment, processes which induce B-lymphocyte maturation and plasma-cell marker expression (Crawford & Ando 1986).

Switching of the latent to lytic cycle is initiated by the EBV immediate early gene products encoded by the open reading frames BZLF1 and BRLF1 (Countryman & Miller 1985, Rooney *et al* 1989, Takada and Ono 1989, Cox *et al* 1990). Following initiation of the lytic cycle, three sets of gene products are expressed in sequential order, referred to as the immediate early, early antigen complex (EA) (Cho *et al* 1985 a & b), and the late genes encoding the viral capsid antigen (VCA) complex (Vroman *et al* 1985) and membrane antigen (MA) complex (Beisel *et al* 1985).

1.3.8.1 Immediate early gene products: BZLF1 and BRLF1

The immediate early genes are expressed early after induction of lytic replication, independently of new protein synthesis, and are required for transactivation of lytic replication (Cox *et al* 1990). Induction of lytic replication by phorbol esters or sIg cross-linking mimics terminal differentiation signals and/ or cause cell cycle arrest (Kieff 1996). The two principal immediate early proteins are encoded by a 1kb mRNA from BZLF1, encoding the protein ZEBRA (also called Z or Zta) and 2.8kb BRLF1 mRNA encoding Rta (Takada & Ono 1989). These genes co-ordinately transactivate early lytic gene expression from two key early promoter regulatory elements within the tandem direct repeat regions DL and DR (duplication L and R). DL and DR encode abundant early mRNAs (Freese *et al* 1983, Dambaugh *et al* 1982, Nuebling & Mueller-Lantzch 1989) and include the origins of lytic viral DNA replication (OriLyt) within the promoter upstream regulatory domain (Hammerschmidt & Sugden 1988). Promoters within DL include those for BHLF1 and BHRF1 (Cox *et al* 1990).

ZEBRA is encoded by a spliced mRNA including three exons (Lieberman & Berk 1990). The protein has homology to c-fos and contains a transactivating domain (Chi & Carey 1993, Flemington *et al* 1992, Lieberman & Burk 1991) and a strongly basic leucine zipper domain which mediates dimerization (Farrell *et al* 1989). The protein functionally and physically interacts with NFκB (Gutsch *et al* 1994) and can downregulate Cp, the latency III promoter, facilitating transition from latency to lytic

replication (Kenney *et al* 1989, Sinclair *et al* 1992). BZLF1 autoregulates its own promoter, Zp, and that of BRLF1 through several AP-1-like binding sites. This mediates promoter activation in the presence of low levels of ZEBRA and repression with high levels of ZEBRA (Taylor *et al* 1991). ZEBRA also binds CREB binding protein (CBP), a histone acetylase and transcriptional coactivator (Bannister & Kouzarides 1996), which leads to dissociation of histones in chromatin and enhances ZEBRA mediated transcription of early promoters (Adamson & Kenney 1999). ZEBRA also exhibits inhibitory effects on Rta's capacity to activate the late gene BLRF2 (Ragoczy & Miller 1999). BZLF1 also induces expression of cyclin dependent kinase inhibitors p21 and p27 and associates with p53, inhibiting p53 induced transcription (Zhang *et al* 1994). Thus ZEBRA promotes growth arrest, but inhibits apoptosis in response to lytic EBV DNA replication (Rodriguez *et al* 1999) and triggers progression through the lytic cycle in both B-lymphocytes and epithelial cells (Cayrol & Flemington 1996a & b, Countryman *et al* 1987, Grogan *et al* 1987). Rta is a sequence-specific DNA transactivator with distant homology to c-myb (Manet *et al* 1991, Gruffat & Sergeant 1994). It interacts directly with Rb (Cannel et *al* 1996) and activates transcription of several genes including EBV DNA polymerase and c-myc and disrupts latency in epithelial cells and certain B-lymphocyte cell lines (Flemington *et al* 1994, Gutsch *et al* 1994, Ragoczy *et al* 1998, Ragoczy & Miller 1999).

1.3.8.2 Early genes

In EBV the early lytic gene complex includes at least 30 proteins encoded by genes intermingled throughout the EBV genome. Most early genes products have enzymatic functions required for viral DNA replication. The early gene products were first characterised from the nuclear and cytoplasmic staining pattern observed using anti-EBV sera against the cell line Raji, which does not express late genes (Henle, Henle & Klein 1971). These early lytic proteins are termed the early antigen complex (EA).

BALF2 is a 135kDa DNA binding protein important in EBV DNA replication (Hummel & Kieff 1982, Fixman *et al* 1992). Also BHRF1, an 18kDa hydrophobic membrane spanning signal peptide with an N-terminal hydrophilic domain and a short C-terminal domain (Pearson *et al* 1987). BHRF1 has homology to bcl-2, is abundantly expressed and may have a role in preventing apoptosis during EBV lytic replication (Henderson *et al* 1993).

Other early genes encode a 117kDa DNA polymerase (BALF5), which associates with several other EBV nuclear proteins (Chiou *et al* 1985, Kiehl *et al* 1991). The EBV DNA polymerase is essential for replication of the EBV episome following induction of lytic replication, which leads to increased numbers of EBV episomes in the infected cells and production of concatomeric linear EBV DNA (Hammerschmidt & Sugden 1988, Pfuder & Hammerschmidt 1996).

EBV also encodes a major DNA binding protein (BALF2), ribonucleotide reductase (BORF2 and BaRF1), Thymidine kinase (BXLF1) and alkaline exonuclease (BGLF1).

1.3.8.3 Late Gene expression

Late gene induction leads to expression of EBV proteins required for structural components of the virion and cleavage of progeny viral genomes, packaging and envelopment of virions and egress of infectious virus particles.

The major nucleocapsid protein p160 is encoded by the BALF4 ORF (Hummel & Kieff 1982). Also amongst the late genes are numerous glycoproteins (gp) including gp350/ 220, encoded by the BLLF1 ORF, which is the most abundant viral protein in the plasma membrane of the lytically infected cell and the viral envelope. Only small amounts are detected in the nuclear membrane, indicating that the virus particle acquires its envelope at the plasma membrane. Other glycoproteins include gp110 (BALF4), one of the most abundant late proteins, and gp85 (BXLF2) (Torrisi *et al* 1989, Gong & Kieff 1990).

BCRF1 encodes a homologue of human IL10 which is 90% similar in amino acid sequence (Hsu *et al* 1990. Vieira *et al* 1991). This may antagonise the effect of

gamma-interferon following cytotoxic inhibition of lytic replication by natural killer cells or cytotoxic T-lymphocytes (Garner *et al* 1984, Koizumi *et al* 1992. Swaminathan *et al* 1993).

1.3.9 *In vitro* EBV infection

EBV is capable of infecting three cell types *in vitro*; resting B-lymphocytes (Pattengale *et al* 1973), thymocytes (Watry *et al* 1991) and squamous epithelial cells (Sixbey *et al* 1983).

1.3.9.1 *In vitro* infection of B-lymphocytes

EBV infects resting B-lymphocytes *in vitro* through attachment of the envelope glycoprotein gp350/220 to the cell surface complement receptor CD21 (or CR2R), expressed on all mature B-lymphocytes (Fingeroth *et al* 1984). Penetration of the cellular membrane and release of the capsid to the cytoplasm involves interaction of additional viral glycoproteins including gp25, gp42 and gp85 with cellular receptors (Li *et al* 1997). The viral genome is transported to the cell nucleus by cytoskeletal transport (Dales and Chardonnet 1973, Keiff 1996). Despite 5% of internalised linear genomes reaching the nucleus (Hurley & Thorley-Lawson 1988), only one copy of the EBV genome is circularised to form an episome and subsequently undergoes amplification to 10-500 clonal copies per cell while the linear genomes are lost (Sugden *et al* 1979).

Immediately following infection, EBV gene transcription is initiated from the Wp promoter, leading to expression of EBNA2 and EBNA- LP, which are detectable by twelve hours post infection (Alfieri *et al* 1991). EBNA2 mediates switching of promoter usage to the Cp promoter, leading to expression of the other EBNA proteins (Woisetschlager *et al* 1989, 1990). EBNA2 also transactivates the cellular genes (CD21, CD23 and c-fgr) and the EBV latent membrane proteins (LMP1, 2a and 2b). EBNA and LMP proteins are detectable by thirty-two hours after infection,

and EBERs transcripts are detectable by seventy hours post-infection (Alfieri *et al* 1991).

Infection of B-cells results in establishment of immortalised LCLs which are continually cycling and capable of proliferating indefinitely (Pope *et al* 1968, Steel *et al* 1977, Tosato *et al* 1985). LCLs phenotypically resemble activated B-lymphocytes, which have been antigenically stimulated and secrete immunoglobulin. LCLs form tumours in T- and B-lymphocyte deficient severe combined immunodeficient (SCID) mice (Rowe *et al* 1991, Bosma *et al* 1983, Mosier 1996). LCLs express cellular activation markers including CD23, CD25 (IL2 receptor), CD30, CD39, CD40, CD70, CD71 (transferrin receptor) and MHCII. The activated blastogenic phenotype is in part induced by LMP1 which mimics constitutively active CD40 leading to upregulation of the cellular adhesion molecules (CAMs) and *bcl*-2 (Thorley-Lawson & Mann 1985, Gregory *et al* 1988).

1.3.9.2 Infection of T-cells *in vitro* and *in vivo*

EBV is associated with a number of T-cell tumours (Jones *et al* 1988, Harabuchi *et al* 1990). Thymocytes, peripheral T-cells and T-cell lines express the EBV receptor CD21 (Tsoukas & Lambris 1988, Fingeroth *et al* 1988, Sauvageau *et al* 1990, Fischer *et al* 1991, Delibrias *et al* 1994) and *in vitro*, EBV can infect thymocytes (Watry *et al* 1991) and T cell lines (Hedrick *et al* 1992, Sinha *et al* 1993). *In vitro* infection of thymocytes leads cellular proliferation and an activated phenotype but the cells are not immortalised (Paterson *et al* 1995). In T-cell lines and T-cell malignancies, EBV expresses a latency II pattern of viral gene expression (Chen *et al* 1993, Minarovits *et al* 1994, Yoshiyama *et al* 1995, Imai *et al* 1996.), that is expression of EBNA1 in the absence of the other EBNAs, and variable expression of LMPs1, 2a and 2b (Kerr *et al* 1992).

1.3.9.3 EBV infection of epithelial cells *in vitro*

Squamous epithelial cell express a homologue of the CD21 EBV receptor (Young *et al* 1989, Birkenbach *et al* 1992). Infection of epithelial cells *in vitro* results

in detection of EBV DNA in differentiated layers and desquamated cells, and expression of EA, VCA or EBNAs (Sixbey *et al* 1983).

1.3.10 *In Vivo* EBV infection

Establishment of a persistent infection is key to the life cycle of EBV *in vivo*. Over 90% of the adult population worldwide is infected with EBV (Henle & Henle 1979). Neonates are protected from EBV infection in the first six months by maternal IgG antibodies (Biggar *et al* 1978) and primary infection generally occurs in childhood, with seroprevalence increasing with age. Initial infection of B-lymphocytes in pharyngeal lymphoid tissues follows transmission of the virus through salivary exchange. In non-industrialised societies, EBV infection generally occurs by three years of age (Henle and Henle 1969, Lang *et al* 1977). Greater concern with hygiene in the industrialised world is responsible for delay in primary infection, which may not occur until adolescence (Henle & Henle 1979). Seroepidemiological studies have demonstrated that there are two peaks in primary infection in the United Kingdom, one occurring between the ages of one and six years, and the second at fourteen to twenty years (Crawford & Edwards 1990). Primary infection is usually asymptomatic, although a self-limiting lymphoproliferative disease, Infectious Mononucleosis (IM), occurs in around 50% of individuals who become infected with EBV in adolescence (Niederman *et al* 1970, Sawyer *et al* 1971). Since infection with EBV is usually asymptomatic, studies on primary infection and the development of immune responses to the virus have generally been carried out on individuals with IM.

1.3.10.1 Infectious Mononucleosis (IM)

IM is commonly known as 'kissing disease', since it is occurs in adolescents following transmission by salivary exchange. Hoagland (1955) suggested a salivary route of transmission for the agent causing IM, but evidence that EBV was the causative agent was gained by chance when a laboratory technician became EBV seropositive in the course of the disease (Henle and Henle 1969). Subsequent studies

of a group of young adults (Niederman *et al* 1970) demonstrated that in subjects who developed IM, antibodies against EBV were consistently absent prior to disease, but appeared during IM and persisted. IM is usually a self-limiting disease which resolves after a few weeks. In some cases however, IM may be chronic or even fatal. EBV is detectable in the saliva of individuals with IM and normal healthy EBV seropositives (Gerber *et al* 1972) and it has also been detected in genito-urinary tract secretions from males and females, indicating the possibility of a sexual route of transmission (Sixbey *et al* 1986, Israele *et al* 1991). The cellular site of EBV replication producing infectious virus is discussed in the next section.

Following virus transmission, EBV infects B-lymphocytes, demonstrated by the ability of B-lymphocytes, obtained during the incubation period or during overt IM, to spontaneously outgrow *in vitro* to produce LCLs and by detection of EBV DNA by PCR in B-lymphocytes (Pope *et al* 1967, Diehl *et al* 1968, Svedmyr *et al* 1984, Neidobitek *et al* 1997). During acute IM, around $2000/10^4$ circulating B-cells in peripheral blood are infected with EBV (Laroche *et al* 1995) and the latency III pattern of gene expression is detected in infected cells (Tierney *et al* 1994, Niedobitek *et al* 1997a). There is also evidence that lytic replication occurs during acute IM; BZLF1 and BCLF1 transcripts have been detected indicating switching to lytic replication (Tierney *et al* 1994, Prang *et al* 1997, Niedobitek *et al* 1997a). Immediate early transcripts and capsid-antigen-encoding transcripts were detected in enriched B-cell populations from 50% and 25% of samples respectively with the corresponding proteins detected in 0.01 to 1.09% of peripheral B-cells (Prang *et al* 1997). EBV DNA has also been detected in the serum of patients with early acute IM, possibly indicating transient viremia (Gan *et al* 1994, Laroche *et al* 1995).

Clinical signs and symptoms of IM include fever, pharyngitis, generalised lymphadenopathy, myalgia, fatigue and in some cases splenomegaly and mild hepatomegaly (Epstein & Crawford 1998). The disease is characterised by lymphocytosis consisting of 'atypical' lymphocytes which are largely antigen-driven oligoclonal CD8+ve T-lymphocytes directed against EBV latent and lytic antigens (Misko *et al* 1980, Callan *et al* 1996). These T cells secrete multiple lymphokines, which are the probable cause of the symptoms of IM (Foss *et al* 1994).

During acute IM, serum IgM and IgG antibodies to VCA and IgG anti-EA antibodies appear early, whereas anti-EBNA1 antibodies do not appear until convalescence (Henle W & Henle 1979). On recovery IgM anti-VCA and IgG anti EA disappear but IgG antibodies to VCA and EBNA1 are maintained for life (Henle G & Henle 1979).

It is not known why primary infection in childhood is generally asymptomatic whereas 50% of adult infections result in IM. This may be due to the dose of virus transmitted, since it is assumed that higher doses of virus are transmitted by salivary exchange between adults by kissing than from transmission to children by aerosol droplets or sucking contaminated objects. It is possible that the EBV-driven B-cell proliferation following a large initial dose may exceed a threshold beyond which the florid T-cell response is activated, resulting in immunopathological effects, and disease. There have also been reports of post-perfusion IM, resulting from transfusion of seropositive donor blood into a seronegative recipient (Gerber *et al* 1969, Blacklow *et al* 1971).

1.3.10.2 Persistent EBV Infection

Like all other human herpesviruses, EBV is able to establish a life-long infection in immunocompetent hosts. Healthy EBV seropositive individuals, regardless of whether their infection resulted from subclinical infection or IM, harbour low numbers of EBV infected B-cells in the circulation, detectable by PCR or their ability to produce LCLs *in vitro*. EBV seropositives also continue to shed infectious virus into the buccal fluid throughout life, detectable by PCR or co-culture of throat-washings with susceptible EBV-negative cord-blood lymphocytes (Yao, Rickinson and Epstein 1985). EBV is detectable in the peripheral blood or throat washings of 94% of EBV seropositive individuals (Faulkner *et al* 1999).

The primary site of persistent infection with EBV is still unclear. Two models have been proposed: persistence in squamous epithelium or in B-lymphocytes, although the balance of evidence supports B-lymphocytes as the reservoir of latent infection.

1.3.10.3 Epithelial cells as the site of latent virus

The hypothesis that epithelial cells act as the source of infectious virus postulates that basal stem cells of squamous epithelium in the oropharynx become latently infected with EBV during primary infection. These cells are self-renewing, and during differentiation, the cells stratify and keratinise, during which EBV undergoes lytic replication and release from the upper layers. This model provides for release of infectious virus into the oropharynx and suggests that B-lymphocytes passing through the epithelium become infected and return to the peripheral circulation and lymphoid tissue (Moss *et al* 1981, Sixby *et al* 1983, Allday & Crawford 1988).

Epithelial cells are infected *in vivo* with EBV in two situations: nasopharyngeal carcinoma (NPC) and oral hairy leukoplakia (OHL). In NPC, EBV DNA is invariably found in malignant cells, and displays a restricted pattern of viral latency. In OHL replicating EBV is detected in differentiated squamous cells layers at the superficial layers of the lesions, but not in the basal layers (Niedobitek *et al* 1991). EBV infected epithelial cells have not been detected in the tonsil of patients with IM or normal individuals, although EBV infected intra-epithelial B-lymphocytes are detected. (Niedobitek *et al* 1992, 1997b, 2000, Anagnostoupoulos *et al* 1995, Tao *et al* 1995, Karajannis *et al* 1997).

1.3.10.4 B-lymphocytes as the site of latent virus

EBV-positive B-cells are dispersed throughout the body, and are detectable in the peripheral blood and within all lymphoid tissues (Nilsson *et al* 1971, Yao *et al* 1985). In the peripheral blood, EBV is found exclusively in resting memory B-cells (Babcock *et al* 1998).

This model places B-cells as the site of persistent infection with EBV, and as source of infectious virus. It postulates that the complete life cycle of EBV is completed within B-lymphocytes. It is proposed that transient latency III EBV gene expression induces infected lymphoblast multiplication, providing a circulating pool of latently infected B-cells. B-cells in the oropharyngeal epithelium also undergo

lytic replication, producing infectious virus which is released into the saliva, providing a route of virus transmission. Virus particles may also infect other naïve B-cells in the submucosal epithelium. A combination of B-cell proliferation and lytic replication could therefore maintain the pool of latently infected B-cells in an individual.

The first direct evidence for B-cells as the site of EBV latency came from the finding that following cytotoxic treatment to destroy haematopoietic tissue prior to bone marrow transplantation in EBV positive individuals, the resident EBV infection is eradicated (Gratama *et al* 1988). If EBV replication is blocked by sustained acyclovir therapy, there is no reduction in the number of latently infected B-cells in the peripheral blood despite a reduction in the levels of oropharyngeal excretion of EBV (Yao *et al* 1989). This suggests that lytic replication is not essential for maintaining EBV infection. EBER positive lymphocytes, but not epithelial cells, have been detected in the nasopharyngeal epithelium and stroma of EBV positive carriers, and in the tonsillar epithelium of patients with IM (Anagnostopoulos *et al* 1995, Tao *et al* 1995). Evidence that B-cells in the epithelium undergo lytic infection has been shown through detection of BZLF1 protein in lymphocytes in the nasopharyngeal epithelium of patients with nasopharyngitis (Senba *et al* 1994).

Further insight into the site of EBV latency came from study by Faulkner *et al* (1999) of patients with X-linked agammaglobulinaemia (XLA), a disorder caused by a mutation in Bruton's tyrosine kinase gene which prevents development of pre-B-lymphocytes into mature functioning B-lymphocytes (Reviewed in Smith *et al* 1998). XLA patients have no antibodies and are therefore particularly susceptible to bacterial infections, but have functional CTL responses against viruses. The 6 patients studied were not infected with EBV, despite infection with the T-lymphotropic herpesvirus HHV-6, indicating that B-lymphocytes are required for infection with EBV.

The precise pattern of EBV gene expression in latently infected cells in healthy EBV seropositives is not clear since the transcripts detected vary between reports, probably due to differences in the sensitivity of the RT-PCR reactions used. Also, RT-PCR of cell populations does not show the pattern of gene expression

within individual cells, rather it shows the combined expression. It is evident that infected cells express a restricted pattern of EBV latent gene expression which does not include the growth-promoting latent genes (Qu & Rowe 1992). The reservoir of EBV infection has been proposed as resting cells in G0 with expression of LMP2a transcripts and variable expression of EBNA1 (Miyashita *et al* 1997). Other reports support the expression of LMP2a and EBNA1 transcripts in normal EBV positives (Qu & Rowe 1992, Tierney *et al* 1994, Chen *et al* 1995) or expression of EBNA1 and LMP1 (Gonella *et al* 1997). Cp and Wp promoter activity is not detected, indicating that the other EBNAs are not expressed. However, in one study, immediate early (BZLF1) and early (BALF2) transcripts were detected in the absence of late BCLF1 transcripts in purified B-cells indicating induction, but not completion, of lytic replication (Prang *et al* 1997).

These studies propose that in primary infection with EBV, the virus infects and activates B-lymphocytes in the oropharyngeal epithelium which become proliferating blasts with full latent viral gene expression and undergo an initial polyclonal expansion to become memory B-cells (Babcock *et al* 1998). The restricted pattern of EBV gene expression in these memory B-lymphocytes allows infected cells to persist in the face of immune responses against latent and lytic antigens. EBNA1 is required for maintaining the EBV genome, and its variable expression may indicate that it is only expressed transiently in dividing cells. Thus an LMP2a-only latency program is proposed as the pattern of gene expression in the true site of EBV persistence (Babcock *et al* 1998). The role of LMP2a in EBV latency is suggested to prevent reactivation of latently infected cells by preventing switching from latent to lytic cycle (Miller *et al* 1994). It is proposed that in lymphoid tissue latently infected cells undergo antigen mediated activation leading to EBV replication or expression of the latency III pattern of unrestricted latent gene expression, and proliferation of EBV infected blasts, which subsequently undergo promoter switching and return to the LMP2a only pattern of gene expression, thus maintaining the pool of latently infected cells.

1.3.11 Host Immune Responses

Despite the potent cell growth transforming potential of EBV, in immunocompetent individuals the virus persists and is maintained as an asymptomatic infection. A balance between virus and host is controlled by immune responses against EBV.

Humoral and cellular immune responses against EBV arise during primary infection, are maintained for life and control life-long infection with EBV. The development of immune responses in primary infection has been studied during IM. Early in IM, antibodies develop against the EA and VCA complexes of proteins. Antibodies against MA also develop, and those against gp350/ 220 are neutralizing (Thorley-Lawson & Geilinger 1980). By the onset of clinical symptoms of IM, high levels of anti-VCA IgM and IgG as well as IgG against EA and MA can be detected. IgA against VCA and EA may also be transiently expressed during this period (Henle & Henle 1979b). IgG antibodies to EBNA1, and less consistently EBNAs 2, 3a, b, c and LP, develop late in IM and levels are often still rising during recovery (Henle *et al* 1987). In addition to the EBV specific antibody response, serum heterophile antibodies can be detected in around 85% of cases of acute IM (Epstein & Crawford 1998). These form the basis of the diagnostic Paul-Bunnel and Monospot tests.

In convalescence from IM and in healthy EBV carriers, stable levels of IgG antibodies are detected against VCA, MA and EBNA1 (Henle & Henle 1979b, Henle *et al* 1987). Neutralizing antibodies against gp350/ 220 may contribute to preventing superinfection with EBV isolates other than the resident virus (Thorley-Lawson and Poodry 1982). 21-30% of virus carriers have IgA antibodies against gp350 in serum, and in 12-19% of carriers these antibodies can be detected in saliva, which may prevent superinfection at mucosal surfaces (Yao *et al* 1991).

The cell mediated immune response is particularly important in controlling EBV infection. A characteristic feature of IM is the presence of high numbers of 'atypical' lymphocytes, predominantly CD8 +ve activated T-cells (Callan *et al* 1996 & 1998). These transiently expanded monoclonal or oligoclonal populations, as determined using monoclonal antibodies or PCR to detect TCR Vβ transcripts (Callan *et al* 1996), recognise EBV antigens and control initial EBV driven B-cell

proliferation. CD4+ve T-cells also contribute to a lesser extent to lymphocytosis in IM (Callan *et al* 1996).

Following primary infection long term memory T-cell responses against EBV latent antigens provide immune surveillance to control persistent infection. CTL responses in healthy carriers are detected by *in vitro* assays against all EBNA proteins with the exception of EBNA1 (Murray *et al* 1988 & 1992, Khanna *et al* 1992, Bogedain *et al* 1995, Levitskaya *et al* 1995, Steven *et al* 1997), which is in agreement with the ability of EBNA1 to inhibit MHCI presentation *in vitro.* In a study of fifteen normal donors, all had CTL responses against at least two latent antigens, with particular prevalence of CTL responses against EBNA3c (Murray *et al* 1990), with less frequent reactivity against EBNA2, EBNA-LP LMP1 and LMP2. Reactivities to particular immunodominant peptide epitopes have been mapped to specific HLA epitopes (Rickinson and Moss 1997).

CTL responses may also be involved in control of the EBV lytic cycle. MHCI restricted CTLs have been detected against the immediate early (BZLF1 and BRLF1) and early (BMLF1, BMRF1 and BALF2) antigens (Steven *et al* 1997). MHCII restricted CD4+ve CTL responses may also have a role in controlling latent and lytic EBV infection since CD4+ve proliferative responses have been detected in normal EBV carriers against EA and gp350/ 220 (Ulaeto *et al* 1988, Pothen *et al* 1991), and against EBNA2 (Khanna *et al* 1997).

1.3.12 EBV Associated Diseases

EBV is associated with a number of human malignancies in the context of persistent infection. These are discussed separately and summarised in Table 1-3.

1.3.12.1 Burkitt's Lymphoma

Burkitt's lymphoma (BL) was the human tumour from which a virus was first isolated (Epstein *et al* 1964a & b), and is the most common childhood malignancy in equatorial Africa. There are two distinct forms of BL, endemic (eBL), which occurs in equatorial Africa and sporadic (sBL), which occurs worldwide at 20 to 100 fold

lower frequency than eBL (O'Conor *et al* 1965). EBV is detected in around 95% of eBL tumours whilst the virus is found in only 1-25% of sBL occurring in Europe and North America (Lenoir *et al* 1986, Magrath 1990) and in less than 50% of AIDS BL tumours (Subar *et al* 1988, Ballerini *et al* 1993). Tumour cells in 97% of cases of EBV positive eBL harbour multiple clonal episomes of EBV, indicating that virus infection occurs before expansion of the tumour cell population (zur Hausen & Schulte-Holthausen 1970, Rowe & Gregory 1989, Neri *et al* 1991). EBV therefore appears to play an important role in BL.

Table 1-3. EBV-associated diseases

Condition	References
African (endemic) Burkitt's lymphoma	Epstein *et al* 1964a & b Zur Hausen *et al* 1970 Neri *et al* 1991
Sporadic Burkitt's lymphomas (a subset)	O'Connor *et al* 1965 Lenoir 1986 Magrath 1990 Gutierrez *et al* 1992
Undifferentiated nasopharyngeal carcinoma	Old et al 1966 Nonoyama *et al* 1973 Wolf et al 1973
Non-Hodgkin's lymphoma in immunosuppressed patients	Crawford *et al* 1980 Peterson *et al* 1985 Purtilo *et al* 1985
Hodgkin's disease (subset)	Weiss *et al* 1989
Oral Hairy leukoplakia	Greenspan *et al* 1985
T/NK cells lymphoma (subset)	Jones *et al* 1988 Harabuchi *et al* 1990
Other tumours with suspected EBV-association: anaplastic gastric carcinoma, salivary gland neoplasia, smooth muscle tumours, breast tumours	Imai *et al* 1994 Raab-Traub *et al* 1991 Lee *et al* 1995 McClain *et al* 1995

BL usually presents in children who are not apparently immunosuppressed and even cases of AIDS associated BL tend to occur early rather than as a late manifestation of AIDS. BL tumours develop extranodaly, commonly in multiple sites, including the jaw, gastrointestinal tract, kidneys, adrenal glands, gonads and bone marrow (Burkitt 1958, Burkitt & O'Connor 1961). BL tumour cells show a germinal centre cell phenotype, and express the common lymphocytic leukaemia antigen CD10 and the BL-associated antigen CD77 (Gregory *et al* 1988, 1990), and cell surface IgM (Pelicci *et al* 1986). BL tumour cells lack lymphocyte activation antigens and cellular adhesion molecules which are characteristic of LCL-like cells. BL tumour cells show a latency I phenotype, in which EBNA1, Bam H1A and EBERs are expressed, although upon explantation *in vitro*, BL tumour cells grow into cell lines which often drift towards a latency III phenotype (Rowe *et al* 1987, Sample *et al* 1991).

BL cells, and cell lines derived from them, consistently display one of three characteristic reciprocal chromosomal translocations: t(8;14), t(8;2), t(8;22), which place the c-myc oncogene under transcriptional control of an immunoglobulin gene locus (Leder *et al* 1983, Klein & Klein 1985). This leads to deregulation and constitutive expression of c-myc (Lenoir *et al* 1986). The characteristic chromosomal breakpoints in the IgH locus on chromosome 14 occur within the JH region in eBL or in the switch (S) region in sBL, suggesting that eBL arises during heavy chain switching in an immature B-cell, and sBL arises in a mature B lymphocyte during Ig class switching.

The observation that eBL geographical distribution coincides with that of holoendemic malaria in Africa and Papua New Guinea suggests that malaria may be a co-factor for eBL pathogenesis. It has been postulated that malaria induces a high level of B-cell turnover, increasing the chance of chromosomal translocation during immunoglobulin gene rearrangement, and EBV infection subsequently confers the final malignant change (Lenoir & Bornkamm 1987). In addition malaria reduces T-cell responses resulting in altered CD4/ CD8 ratios (Whittle *et al* 1984). Increased numbers of EBV infected B-lymphocytes are detected in individuals with malaria (Lam *et al* 1991) and EBV may drive an initial B-cell proliferation as a result of

malaria induced immunosuppression which increases the chance of chromosomal translocation, and emergence of a malignant clone (Klein 1987).

1.3.12.2 Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is another EBV associated malignancy which shows a distinct geographical distribution. It is a squamous epithelial tumour which occurs at a rate of less than 1 in 10^5 of the population in Europe and the USA, whereas the incidence in South China and South East Asia is 100-fold higher. An intermediate incidence is seen in Inuits of North America and Greenland and in certain North African populations (Shanmugaratnam 1971, 1978). The differences in incidence rate worldwide probably reflect genetic predisposition in different racial groups (Simons *et al* 1974, Chan *et al* 1983) and the influence of local environmental or dietary factors. Substances that have been identified as potential co-factors in NPC development include carcinogenic nitrosamines in traditional salted fish (Huang, Ho & Gough 1978, Shao *et al* 1988) and phorbol esters in traditional Chinese medicines taken as snuff (Hirayama & Ito 1981) and domestic wood smoke (Zheng *et al* 1994).

NPC tumours are undifferentiated in type in around 70% of cases, the remainder showing squamous differentiation. Tumour cells in undifferentiated NPC are consistently EBV positive, regardless of the population in which they occur (Klein 1979, Niedobitek *et al* 1991, Raab-Traub 1992), and tumours characteristically show a latency II pattern of EBV gene expression (ie EBNA1, and variable levels of LMP1 and LMP2a or b) (Young *et al* 1989, Brooks *et al* 1992). NPC tumours show a characteristic histological appearance, with considerable infiltration of the tumour mass with non-malignant CD4+ve T-lymphocytes. This appears not to be a specific immune response, rather a response to lymphokine release by the tumour cells (Busson *et al* 1987). Characteristic serum antibody profiles are detected in patients with NPC which is effective in diagnosis and screening of susceptible populations (Zeng *et al* 1982). Serum IgG and IgA antibodies against VCA and EA-D are raised and IgA against these antibodies is detected in saliva (Klein 1979) Detection of anti-VCA IgA suggests that lytic replication at a mucosal site precedes NPC development. However, full lytic

replication has not been demonstrated in NPC, despite detection of the lytic transactivator BZLF1 in a few tumour cells (Cochet *et al* 1993).

The pathogenesis of NPC raises questions about infection by EBV of epithelial cells. It has been proposed that the anti-EBV IgA responses may facilitate entry of EBV to epithelial cells by transport of IgA complexes across epithelial membranes (Sixbey & Yao 1992).

1.3.12.3 Hodgkin's Disease (HD)

HD is the most common malignant lymphoma in the Western world with two peaks of incidence occurring at 15-35 years and over 50 years of age. The socio-economic epidemiology of HD mirrors that of IM, and there is a 4- to 6-fold increase in the incidence of HD within 5 years of IM (Munoz *et al* 1978, Bernard *et al* 1987, Mueller *et al* 1989). EBV DNA is present in tumour cells in around 60% of cases of HD, localised to the malignant Reed-Sternberg (RS) cells and Hodgkin's cells which are characteristic of HD, and are proposed to be of immature B-lymphoid origin (Anagnostopoulos *et al* 1989, Weiss *et al* 1989, Herbst & Niedobitek 1994). Southern blot analysis of the EBV terminal repeat region has demonstrated monoclonality of EBV episomes in HD (Anagnostopoulos *et al* 1989, Gulley 1994) EBV in HD shows a Latency II pattern of EBV gene expression (EBNA1, LMP1 and LMP2a and b) (Herbst *et al* 1991, Pallessen *et al* 1991, Deacon *et al* 1993, Grasser *et al* 1994, Niedobitek *et al* 1997). There is some evidence for a role of genetic changes in HD, including mutation of p53 (Gupta *et al* 1993, Niedobitek *et al* 1993) and Rb (Morente *et al* 1997).

HD tumours are divided into four groups according to the Rye classification: lymphocyte predominant, lymphocyte depleted, nodular sclerosing and mixed cellularity (Rappaport *et al* 1971). Over 80% of the more aggressive mixed cellularity and lymphocyte depleted forms and around 30% of nodular sclerosing HD are EBV positive, and less than 10% of lymphocyte predominant HD are EBV positive (Herbst *et al* 1990, 1992).

1.3.12.4 T-cell lymphomas

Although T cells have been shown to express the EBV receptor CD21 (Fisher *et al* 1991), EBV infection of T-cells is uncommon (Niedobitek *et al* 1992, Anagnostopoulos 1995). However a number of EBV associated malignancies of T-cell origin have been described, although the pathology of EBV associated T-cell neoplasia is unclear. EBV is associated with 84% of nasal T-cell lymphomas (midline granuloma) in adults (Harabuchi *et al* 1990), up to 97% of cases of peripheral T-cell lymphomas of angioimmunoblastic lymphadenopathy type (Ott *et al* 1992), and 46% of peripheral T-cell lymphomas (Jones *et al* 1988). In peripheral T-cell lymphomas and midline granuloma, EBV shows a latency II phenotype, as seen in NPC and HD (Minarovits *et al* 1994).

1.3.12.5 Oral Hairy Leukoplakia (OHL)

Oral hairy leukoplakia is a benign superficial epithelial lesion mainly of the lateral boarders of the tongue which occurs in immunosuppressed individuals (mostly HIV positive patients). Epithelial cells in the outer, more differentiated layers, but not the basal layers contain EBV DNA and express EBNA1 (Thomas *et al* 1991). The lesions mark a site of EBV replication (Greenspan *et al* 1985) although the viral genome has not been detected in the basal layers of the lesion (Niedobitek *et al* 1991b, Thomas *et al* 1991). OHL responds to acyclovir treatment, and recurs when it is withdrawn, demonstrating the role of lytic replication in the lesion (Resnick *et al* 1988).

1.3.12.6 AIDS related lymphoma

Up to five percent of AIDS patients develop lymphoma, 60% of which are immunoblastic lymphomas (aIL), 21% are Burkitts lymphoma (aBL), and 19% primary AIDS related central nervous system (CNS) lymphomas (aCL) (Beral *et al* 1991, Herndier *et al* 1994). EBV is associated with virtually all aCLs and around 80% of aILs, whereas only around a third of aBL are EBV associated (Hamilton-Dutoit *et al* 1993a & b). aIL lesions show latency I, II or III phenotypes and a

proportion of them show early lytic gene expression (Pallessen *et al* 1991b, Hamilton-Dutoit *et al* 1993b).

1.3.12.7 X-linked lymphoproliferative syndrome (X-LPS)

X-LPS was first identified following a report of fatal acute IM or malignant lymphoma in six male kindred of the same family (Purtillo *et al* 1974, 1975). The syndrome is caused by a defective gene on the X-chromosome called SAP (Signalling lymphocytic activation molecule (SLAM) associated protein), which is an inhibitor of T-cell signal transduction following ligand binding, functioning to modulate T-cell activation (Sylla *et al* 2000, Sayos *et al* 1998). It is thought that loss of SAP function results in an uncontrolled CTL response following primary infection with EBV and inability to control B-cell proliferation caused by EBV (Skare *et al* 1987). This results in fulminant and rapidly fatal acute IM or less commonly chronic disease which often progresses to fatal B-cell lymphoma. Death occurs in all cases by 40 years of age, most commonly due to vital organ failure caused by tissue destruction caused by the uncontrolled CTL response (Grierson & Purtillo 1987). X-LPS is characterised histologically by solid organ lymphoproliferations of EBNA positive B lymphoblastoid/plasmacytoid cells of polyclonal origin mixed with T, NK cell and phagocytic cells (Purtillo *et al* 1981a & b, Mroczek *et al* 1987, Thomas *et al* 1991c).

1.3.12.8 Post Transplant Lymphoproliferative Disease (PTLD)

PTLD is a rare but frequently fatal complication of iatrogenic immunosuppression. The term PTLD encompasses a spectrum of B-cell lymphoproliferations ranging from reactive plasmacytic hyperplasia to monomorphic B-cell lymphoma (Harris, Ferry and Swerdlow 1997). The tumours are almost always associated with EBV, and the malignancies reflect an imbalance in the normal control of EBV infection. The disease is characterised by rapid onset, aggressive behaviour and high mortality, even with treatment.

1.3.12.8.1 Incidence

Iatrogenic immunosuppression following organ transplantation predisposes patients to a variety of cancers (Hanto *et al* 1981). The incidence of NHLs in transplant recipients, termed PTLD, is 28 to 100-fold higher than that observed in age-matched controls (Kinlen *et al* 1979, Penn 1993). The incidence of PTLD varies with transplant type and in different transplant centres (Nalesnik 1998). Reported incidence of PTLD in kidney transplant recipients is 0.4 - 2.5% (Birkeland 1983, Starzl *et al* 1984), 2.3 - 13.7% in liver (Renard *et al* 1991, Cox *et al* 1995), 0.2 - 1.6% in bone marrow (Beveridge *et al* 1984, Shapiro *et al* 1988), 1.8 - 3.4% in heart (Nalesnik *et al* 1988, Armitage *et al* 1991) and 4.6 - 9.4% in heart/ lung recipients (Nalesnik *et al* 1988, Randhawa *et al* 1989).

1.3.12.8.2 Risk Factors

The development of PTLD is thought to be the result of interplay of a number of factors. These include genetic predisposition (Penn 1979), defective humoral and cellular immunity caused by primary disease (Sheil 1986), chronic antigenic stimulation by the allograft (Gleichmann *et al* 1975), impaired immunosurveilance (Klein & Klein 1977), and long term immunosuppression (Calne *et al* 1979, Nagington & Gray 1980). The principal risk factors for PTLD development are duration and degree of iatrogenic immunosuppression given to prevent graft rejection, and primary EBV infection following transplantation. Paediatric transplant recipients are more frequently EBV seronegative, and in a retrospective serological study, the majority of children developing PTLD had undergone primary EBV infection in the six months prior to developing PTLD (Ho *et al* 1985, 1988, Penn 1991, Sokal et al 1993, Thomas *et al* 1995).

The introduction in 1979 of cyclosporin A (CsA) as the principal immunosuppressive drug used to prevent graft rejection was accompanied by a large increase in the incidence of PTLD (Penn 1988). The commonly used triple therapy approach of combined CsA, azathioprine (AzA) and prednisolone has been associated with an increase in NHL in kidney and heart transplant recipients

(Wilkinson *et al* 1989). Increased incidence of PTLD has also been associated with use of more potent and specific immunosuppressive drugs including antithymocyte globulin (ATG) (Sokal *et al* 1997), FK506 (Nalesnik *et al* 1991, Shapiro *et al* 1999) and the murine anti-CD3 monoclonal antibody OKT3 (Swinnen *et al* 1990). The risk of PTLD is also increased in patients who undergo retransplantation, due to the increased cumulative dose of immunosuppression (Weintraub & Warnke 1982, Opelz & Henderson 1993).

In a multicentre study with a ten year follow-up of 45,141 kidney and 7,634 heart transplant recipients, both groups had a high incidence of PTLD in the first year post-transplant (0.2% of kidney and 1.2% of heart transplant recipients, which is 20 and 120 times higher than the incidence in the general population respectively) (Opelz & Henderson 1993). This coincides with the period of the most aggressive immunosuppressive therapy, and the incidence of PTLD reduced to 0.04% and 0.3% per year in kidney and heart recipients respectively in subsequent years, reflecting the reduced level of maintenance doses of immunosuppression. The highest rates of PTLD incidence are in heart, lung and liver transplant recipients, who receive the highest doses of immunosuppression required to prevent life-threatening graft rejection.

1.3.12.8.3 Clinical Presentation

The diversity of clinical and histological presentation of PTLD makes diagnosis difficult, and the course of the disease is frequently aggressive and fatal.

PTLD most commonly occurs in the first year following transplantation. This "early onset" PTLD frequently presents as an IM-like illness with localised or generalised lymphadenopathy (Nalesnik *et al* 1998). This presentation is most commonly seen in patients who are EBV seronegative pre-transplant and 50% of cases of PTLD develop in transplant recipients who had undergone primary infection with EBV in the previous six months (Ho *et al* 1985, Thomas & Crawford 1990). PTLD in EBV seropositive transplant recipients tends occur over one year post transplant, and may develop after many years. These 'late onset' tumours tend to be

extranodal, arising as localised tumours in single or multiple sites (Ho *et al* 1988). Extranodal lesions occur in 69% of cases and 51% of lesions have been found in multiple organs (Penn 1993). PTLD commonly develops in sites that are unusual in the context of other lymphomas including the central nervous system, gastrointestinal tract or grafted organ. Lesions develop in the CNS in 12% of cases of PTLD, compared with 1-2% of lymphomas in the general population (Penn & Porat 1995). Graft involvement is most common in lung transplant recipients, probably due to presence of lymphoid tissue in the lung which is not present in other grafted solid organs (Nalesnik 1998, Thomas & Crawford 1990, Penn and Porat 1995).

1.3.12.8.4 Classification

PTLD is usually of B-lymphocyte origin, although some T-cell lymphomas have been reported (Penn 1994, Leblond *et al* 1998). PTLD lesions can be polyclonal, oligoclonal or monoclonal, on the basis of cell surface and cytoplasmic Ig expression or Ig gene rearrangements (Frizzera *et al* 1981, Hanto *et al* 1982, Cleary & Sklar 1984, Knowles *et al* 1995). Individual lesions arising in the same patient can show diverse clonality, containing poly-, oligo- or mono-clonal B-lymphocytes (Shearer *et al* 1985). Assessment of tumour cell clonality is of limited use in providing clear prognostic information in PTLD due to the unpredictable nature of these lesions. However, polyclonal lesions tend to be more responsive to conservative treatment such as reduction of immunosuppression (See Treatment section 1.3.12.8.7) whereas monoclonal tumours are often more aggressive. Progression of PTLD from polyclonal to more aggressive monoclonal lesions regularly occurs in untreated cases or in recurrent lesions (Nalesnik 1998), and probably involves additional cellular genetic changes, although this appears to be inconsistent feature (Locker and Nalesnik 1989). There have been reports of changes in the tumour suppressor gene p53 and oncogenes c-myc and N-ras (Knowles *et al* 1995, Chadburn *et al* 1997). An unusual EBV positive BL-like PTLD has been reported in a heart transplant recipient, which carried the characteristic t8;14 c-myc translocation (Hunt *et al* 1996).

PTLD lesions have recently been classified into five groups on the basis of tumour histology and presentation (Harris, Ferry and Swerdlow 1997). This classification is summarised in Table 1-4.

Table 1-4 Histological classification of PTLD lesions (Harris, Ferry and Swerdlow 1997)

PTLD Type	Histological Classification
1	Early lesions including IM-like reactive plasma cell hyperplasias
2	Polymorphic PTLD which may be polyclonal or monoclonal
3	Monomorphic PTLD of diffuse large B-cell lymphoma (immunoblastic, centroblastic or anaplastic) or Burkitt's type
4	T-cell lymphoma
5	Other, including Hodgkin's disease, plasmacytoma-like lesions and myeloma

1.3.12.8.5 EBV Association

Involvement of EBV in PTLD was demonstrated by detection of EBV antigens in tumour tissue sections (Crawford *et al* 1980) and detection of EBV DNA in tumour cells (Weiss & Mohaved 1989). The detection of clonal EBV in monoclonal PTLD suggests that EBV infection occurs at an early stage in PTLD development before expansion of the tumorigenic clone (Patton *et al* 1990).

PTLD is EBV associated in the majority of tumours of B-cell origin, and one-third of T-cell cases (Young *et al* 1989, Thomas *et al* 1990, Dockrell *et al* 1998). However in one report eleven of thirty-four cases (32%) of post-solid organ transplant NHL were EBV negative (Leblond *et al* 1998). These cases were late onset, many occurring over five years post-transplant.

In solid organ transplant recipients who are EBV negative pre-transplant, the donor organ can act as the source of infectious virus (Cen *et al* 1991, van Gelder *et al* 1994, Haque *et al* 1996). In bone marrow transplant recipients, bone marrow destruction prior to transplantation can result in loss of the recipients original EBV

isolate, which can be followed by transmission of a new EBV isolate in transplanted bone marrow (Gratama *et al* 1988, 1990). Ablation of the recipients' bone marrow pre-transplant and reconstitution of the immune system with donor cells means that PTLD is usually of donor cell origin (Shapiro *et al* 1988).

The serological profile of primary infection in transplant recipients differs from that seen in normal healthy individuals, is often of little value in the diagnosis of PTLD and may be misleading due to the severe immune dysfunction of these patients. IgM responses to VCA heterophile antigen are not reliably detected (Henle & Henle 1981, Thomas & Crawford 1990). Patients who are EBV carriers pre-transplant commonly show a serological profile suggestive of a viral reactivation, diagnosed by raised levels of IgG against VCA and EA but not to EBNA (Henle & Henle 1981, Ho *et al* 1985, Thomas & Crawford 1990).

EBV gene expression in PTLD tumours is typically latency III, with LCL-like expression of all latent genes (Young *et al* 1989c, Thomas *et al* 1990). In some cases latency I or II are seen, and EBV gene expression within a tumour may be heterogeneous (Cen *et al* 1993, Neidobitek *et al* 1997). In a minority of cells within the tumour there is evidence of EBV lytic replication by detection of linear EBV DNA and immediate early transcripts, and less commonly early and late viral proteins (Katz *et al* 1989, Rea *et al* 1994). Tumour cells express B cell activation markers and cellular adhesion molecules (CAMs) (Young *et al* 1989c, Thomas *et al* 1990).

1.3.12.8.6 Pathogenesis

In vitro experiments have shown that immunosuppressive drugs are potent suppressors of EBV specific CTLs (Crawford *et al* 1981, Tsoukos *et al* 1982, Kino *et al* 1987, Burman & Crawford 1991). *In vivo*, EBV-specific CTL activity becomes low or undetectable in all EBV seropositive transplant recipients following transplantation (Haque *et al* 1997). Loss of CTL activity creates a favourable environment for EBV infected cells to persist and express additional latent and lytic proteins without elimination by EBV-specific CTLs.

Bone marrow transplant recipients frequently have underlying immunodeficiencies which represent an independent risk factor for PTLD in this group (Bhatia *et al* 1996) and following transplantation are severely immunodeficient due to destruction of their bone marrow by chemotherapy or radiotherapy and through immunosuppression to prevent graft versus host disease (GVHD). The risk of PTLD in bone marrow transplant recipients is increased if the donor marrow is depleted of T-cells prior to infusion to reduce GVHD since HLA-matched donor T-cells would be capable of mounting a defence against development of PTLD of donor B-cell origin (Shapiro *et al* 1988). However, if B-cells are removed from the graft before transplant (Cavazzana-Calpo *et al* 1998) or if the donor marrow is depleted of T-cells and coincidentally of B-cells by counterflow elutriation this risk factor is reduced (Gross 1998).

Following transplantation increased levels of EBV are shed in saliva and higher concentrations of EBV infected B-lymphocytes and EBV DNA have been detected in the circulation (Wagner *et al* 1992, Preiksaitis *et al* 1992, Savoie *et al* 1994, Riddler *et al* 1994, Kenagy *et al* 1995, Rowe *et al* 1997). High levels of EBV are thought to reflect the loss of immune control of EBV infection, with uncontrolled proliferation of EBV infected B-cells. This is termed a 'reactivated' EBV infection, although in most cases no disease ensues. However in a minority of patients it is thought that uncontrolled EBV-driven B-cell proliferation leads to PTLD. In an EBV seronegative transplant recipient, an efficient CTL response cannot be established if primary infection with EBV occurs post-transplantation, and it is thought that proliferation of infected B-cells leads to the polyclonal immunoblastic lesions seen in early onset PTLD. Although high levels of EBV DNA have been detected in most cases of PTLD, this is not always the case, and similar levels can be detected in patients who do not develop disease (Savoie *et al* 1994, Kenagy *et al* 1995). EBV DNA has also been detected in the serum of patients with PTLD, indicating that some cells undergo lytic replication in PTLD (Scichman *et al* 1998, Limaye *et al* 1999). EBV DNA has previously been detected in the serum of patients with IM (Gan *et al* 1994) and NPC (Yamamoto *et al* 1995). Babcock *et al* (1999) reported that increased EBV load following transplantation is due to an increase in the number

of circulating latently infected resting memory B-cells or lytic replication rather than proliferating lymphoblasts. A model for PTLD development in the context of normal latency is described in the Discussion, section 4.3.3.

In tumour biopsy material, EBV positive cells may make up only a small proportion of the tumour mass, with an extensive EBV negative infiltrate comprising predominantly CD4+ve T-lymphocytes (Perera *et al* 1998). *In vivo* experiments in SCID mice, where PTLD-like tumours can be generated following injection of human peripheral blood mononuclear cells, show that T-cells are necessary for development of these tumours (Veronese *et al* 1992). This suggests a role for the non-tumour element in PTLD development, possibly in supplying essential growth factors. RT-PCR and *in-situ* hybridisation studies of SCID tumours have demonstrated that tumour cells produce cytokines which stimulate B-lymphocytes in an autocrine fashion (Johannesen & Crawford 1999). It is likely that additional factors are involved in rendering particular organs more favourable as sites for PTLD development. Occurrence of PTLD in the transplanted organ, for example, may reflect the complex immunologic interactions taking place during graft rejection or dysfunction, which can enhance cytokine production and possibly aid tumour development (Tosato *et al* 1993, Wakasugi *et al* 1987).

A recent study suggests an association between PTLD and CMV disease (Manez *et al* 1997). This may reflect the combined loss of immune control for EBV and CMV with high dose immunosuppression, but it is also possible that reactivation of CMV has profound effects on the immune system by inducing cytokine production which directly transactivates EBV genes (Aalto *et al* 1998).

1.3.12.8.7 Treatment

Despite the use of a variety of treatment strategies, mortality from PTLD remains high, and can be up to 70% (Armitage *et al* 1991). Published studies tend to be small scale, anecdotal and complicated by the diversity in presentation of PTLD and histological differences between cases. In general however, since the observation by Startzl *et al* (1984) that lesions could regress spontaneously following reduction in

immunosuppression, this is now the first line of treatment for PTLD in solid organ transplant recipients. There is no definitive classification for predicting the response of these tumours to reduced immunosuppression, although early onset polyclonal PTLD associated with primary EBV infection is usually more responsive than monoclonal tumours appearing in EBV seropositive individuals over one year post-transplant (Nalesnik *et al* 1998, Chadburn, Cesarman and Knowles 1997, Harris *et al* 1997). Clearly this strategy carries with it the risk of graft rejection, so cases must be carefully monitored and treatment tailored to clinical events. High doses of the antiviral drugs acyclovir or ganciclovir, which inhibit EBV DNA polymerase and lytic replication (Colby *et al* 1980, Datta *et al* 1980, Collins *et al* 1983) are often included as a treatment in combination with reduced immunosuppression (Hanto *et al* 1982, Pirsch *et al* 1989, Davis 1998). However, since these drugs only block herpesvirus replication their value in PTLD, where most EBV infected cells are latently infected, remains unclear. There have been reports of EBV negative patients acquiring the virus after transplant and developing PTLD whilst still on high dose acyclovir (Haque *et al* 1996)

Surgical resection, irradiation or chemotherapy are used to treat PTLD where initial reduction in immunosuppression fails (Gross *et al* 1998b, Hanto *et al* 1983). In one study a 75% complete remission rate was achieved using a multiple cytotoxic drug regime in eight heart-transplant patients who presented with monoclonal PTLD refractory to reduction of immunosuppression (Swinnen *et al* 1995). Interferon alpha has also been effective at inducing complete remission in both polyclonal and monoclonal lesions although the precise mode of antitumour action is not known (Davis *et al* 1998, Faro 1998). There has also been success in using monoclonal antibodies against the B-lymphocyte surface markers CD21 and CD24 to treat PTLD (Benkerrou *et al* 1998, Fischer *et al* 1991).

New treatment strategies involving adoptive T-cell immunotherapy are now being assessed since PTLD presents an ideal target for this type of therapy for several reasons (Haque & Crawford 1999). Firstly, immunogenic EBV antigens are expressed only on tumour cells and not on normal cells, so the therapy is specific for the tumour. Secondly, CTLs are relatively easy to culture *in vitro* to high numbers.

Thirdly, immunotherapy carries less risk of damage to the grafted organ than reduction of immunosuppression. This is of particular importance in heart/lung and liver transplants where loss of the graft would be fatal. Finally, immunotherapy is non-toxic and therefore preferable to chemotherapy.

EBV specific CTL activity can be restored in bone marrow transplant recipients through adoptive transfer of EBV specific polyclonal T-cells of donor origin (Rooney *et al* 1995). A pilot study has demonstrated restoration of EBV specific CTL activity in healthy EBV seropositive liver and kidney transplant recipients following infusion of *in vitro* expanded autologous CTLs (Haque *et al* 1998). The CTLs were retained, remained functional for at least 3 months post-infusion, and caused reduction in levels of EBV DNA detectable in the blood. Nalesnik *et al* (1997a & b) reported regression of EBV positive PTLD in four solid organ transplant recipients following treatment with autologous mononuclear cells which had been depleted of monocytes and stimulated *in vitro* with IL2 for ten to eleven days prior to infusion, producing lymphokine activated killer cells (LAK cells). Advances have also been made in using chemically engineered major histocompatability complex tetramers which could be used to enrich populations of antigen specific CTLs (Altman *et al* 1996). These complexes have already been used by Callan *et al* (1998) to directly visualize antigen specific CD8 +ve T-cells during a primary immune response to EBV *in vivo* in humans.

The strategy for adoptive CTL therapy in bone marrow transplant recipients is more straightforward than in solid organ transplant recipients, since in most cases the marrow donor is available. As the recipient has adopted donor lymphoid tissue, and any developing PTLD is generally of donor cell origin, CTLs obtained from the donor can be used for therapy. In solid organ transplant recipients however, where the organ is usually of cadaveric origin, autologous CTLs must be grown *in vitro* from T-cells obtained pre-transplant for use at a later date if PTLD should arise. This would be a costly and time-consuming procedure since ideally T-cells should be grown for each transplant recipient. An alternative strategy is to use allogeneic CTLs grown from healthy donors on a best HLA-match basis (Trainor *et al* 1991). This strategy is now being developed by Dr Tanzina Haque in our laboratory such that a

panel of frozen HLA typed EBV specific CTLs is banked and ready to infuse into patients with PTLD.

1.3.12.8.8 Prevention of PTLD

The transfer of donor EBV isolates to an EBV negative recipient must initially involve EBV lytic replication to release infectious virus and infect the new host. This could be avoided by matching EBV status of donor and recipient. This is impractical due to the urgency of transplantation and scarcity of EBV seronegative donors. An alternative approach could be to prevent infection of the recipient using neutralizing antibodies against the EBV envelope glycoprotein gp340 which would prevent virus binding to the cell surface receptor CD21. A vaccine to induce antibodies against gp340 is under production, although clinical trials are not yet completed. Alternatively EBV negative transplant recipients could be passively immunized with neutralising anti-gp340 monoclonal antibodies to prevent infection in the critical period of intense immunosuppression when there is high risk of PTLD. A pilot study using this approach is currently underway in paediatric liver transplant recipients (Personal communication: Haque, Crawford and Mieli-Vergani).

Another approach would be to use a peptide-based vaccine to induce EBV epitope-specific CTL responses in EBV seronegative transplant recipients. The choice of peptide vaccine would be tailored to the HLA Class I allele of individual recipients or alternatively multiple CTL epitopes could be combined to cover the HLA restriction of up to 80% of the Caucasian population (Moss *et al* 1998). Alternatively a DNA plasmid vaccine could be used which encodes multiple minimal CTL epitopes. This approach has proved effective at inducing protective CTL epitopes in mice (Thomson *et al* 1998).

Early identification of patients at risk of developing PTLD is crucial to the success of any therapeutic approach. Increased EBV load has been cited as a predictive marker of PTLD development, and it has been suggested that transplant recipients should be monitored for sustained elevation of viral load (Stevens *et al* 1999, Swinnen 2000). However, since not all patients with high EBV load go on to

develop PTLD (Savoie *et al* 1994, Kenagy *et al* 1995. Rowe *et al* 1997), a more reliable method of predicting PTLD is required.

2 AIMS

This study set out to monitor the course of EBV infection in adult cardiothoracic transplant recipients in order to relate changes in EBV copy number and EBV gene expression to development of PTLD.

3 Materials and Methods

3.1 Reagents

Reagent	Supplier
1Kbp DNA ladder	Gibco BRL
^{32}P-γ-dATP	Amersham International
Bovine serum albumin (BSA)	Sigma
Bromophenol blue	Sigma
DNA polymerisation mix	Pharmacia biotech
Ethidium Bromide (EtBr)	Gibco BRL
Ficoll-400	Pharmacia Biotech
Ficoll-Hypaque	Pharmacia Biotech
Foetal calf serum (FCS)	Sera Lab
Formamide	Gibco
Glycerol	Sigma
Mineral oil	Sigma
Mixed bed resin	Sigma
NICK™ sepharose columns	Pharmacia Biotech
NuSieve 3:1 agarose	FMC BioProducts
Oligonucleotides	Oswel
RNAzol™ B	Cinna/ Tel Test Inc
RT-PCR kit	Stratagene
Sheared salmon sperm DNA	5'-3' Inc
T4 Polynucleotide kinase	Promega
Taq DNA polymerase	Promega
Tissue culture media	Gibco BRL
Trypan Blue	Flow
X-ray fixer and developer	Photosol
ϕX174 DNA/ *Hae* III markers	Promega

3.2 Suppliers

5'-3' Inc, Boulder, Colorado, USA

American National Can, Neenah, Wisconsin, USA

Amersham International Plc, Little Chalfont, Buckinghamshire, UK.

Anachem, Luton, Bedfordshire, UK.

BDH Ltd, Poole, Dorset.

Beckman Instruments, Palo Alto, California, USA.

Beckton-Dickinson Ltd, Cowley, Oxfordshire, UK.

Bibby Sterilin Ltd, Stone, Staffordshire, UK.

Bio-Rad laboratories Ltd, Hemel Hempstead, Hertfordshire, UK.

Cinna/Tel-Test Inc., Friendswood, Texas, USA.

ECACC, Salisbury, Wiltshire, UK.

Flow-ICN (UK), Thame, Oxfordshire, UK.

Flowgen, Lichfield, Staffordshire, UK.

FMC Bioproducts, Rockland, Maine, USA.

Gibco BRL, Paisley, Scotland

Heraeus, Brentwood, Essex, UK.

Hybaid Ltd, Teddington, Middlesex, UK.

Invitrogen Corporation, San Diego, California, USA.

Jencons, Leighton Buzzard, Bedfordshire, UK.

Marathon Laboratory Supplies (Falcon), London, UK.

MSE (Sanyo-Gallenkamp), Uxbriodge, Middelsex, UK.

Nunc, Naperville, Illinois, USA.

Oswel DNA services, Southampton, Hampshire, UK.

Perkin-Elmer Cetus, Beaconsfield, Bedfordshire, UK.

Pharmacia Biotech, Uppsala, Sweden.

Phillip Harris, London, UK.

Photosol, Basildon, Essex, UK.

Promega, Madison, Wisconsin, USA.

Scotlab, Coatbridge, Lancashire, UK.

Sera-lab, Crawley Down, West Sussex, UK.

Shandon Scientific, Runcorn, Cheshire, UK

Sigma Chemical Corporation, St Louis, Missouri, USA.

Sterilin, Hounslow, Middlesex, UK.

Stratagene Cloning Systems, La Jolla, California, USA.

UVP Inc., San Gabriel, California, USA.

Whatman, Maidstone, Kent.

3.3 Consumables

Item	Supplier
3MM chromatography paper	Whatman
Cell culture flasks and plates	Marathon (Falcon)
Cryotubes	Nunc
Disposable gloves	Johnson and Johnson Medical Inc
Dry ice (solid CO_2)	Distillers MG
Filter Units (0.45μm, 0.60μm)	Millipore
Hybond N+ nylon membrane	Amersham International Plc
Hyper-MP film	Amersham International Plc
Microfuge tubes (0.5ml/ 1.5ml)	Scotlab
Micropipette tips	Anachem
Parafilm	American National Can
Plastic bijoux	Sterilin
Plastic universal tubes (25ml)	Sterilin
Polypropylene conical tubes (50ml)	Marathon (Falcon)
Saran wrap	Genetic Research Instrumentation
Syringes	Philip Harris

3.4 Equipment

Item	Model and supplier
Automatic pipettor	'Pipette-boy', Arnold R Horwell
Centrifuge	'Omnifuge 2.0 RS0', Heraeus
	'Microcentaur', MSE
CO_2 incubator	'CO_2 auto-zero', Heraeus
Film cassettes	Genetic Research Instrumentation
Film Developer	Fuji Photo Film Company
Geiger counter	'Mini-monitor 900 GM type E', Mini-Instruments
Gel Documentation System	UVP with Phoretix 2D advanced scanning densitometry analysis software
Gel electrophoresis tanks	Gibco BRL
Haemocytometer	'New Improved Neubauer', BDH
Heating block	Stuart Scientific
Hotplate	Gibco BRL
Hybridisation oven	Hybaid
Magnetic stirrers	BDH
Microbiological safety cabinet	'Micrflow class 2' Heraeus
Microscopes	Leitz, Olympus
Microwave	Sharp
PCR machine (thermal cycler)	'Omnigene', Hybaid
pH meter	Philip Harris
Pipettes	Gilson, Anachem
Power pack	'2197', Pharmacia Biotech
	'500/200' Bio-Rad
Refrigeration	-70°C, Philip Harris Scientific
	-20°C, Electrolux
	+4°C, Electrolux
UV Stratalinker	'Stratalinker 2400', Stratagene
Vortex	'Vortex-Genie', Scientific Industries

Waterbaths	Grant Intruments

3.5 Materials and Solutions

Cell culture

Culture Medium

(made up in 1X RPMI 1640)

L-glutamine	2mM
Penicillin	100U/ml
Streptomycin	100μg/ml
Foetal calf serum	10% v/v

Wash Medium

(Made up in sterile dH_2O)

Penicillin	100u/ml
Streptomycin	100μg/ml
Foetal Calf serum	2% v/v
7.5% Sodium Bicarbonate	2.7% v/v
1M Hepes buffer	2% v/v
10X RPMI 1640	10% v/v

Freezing Medium

Foetal Calf serum	90% v/v
Dimethylsulphoxide (DMSO)	10%v/v

Phosphate Buffered Saline (PBS)

NaCl	0.8% w/v
KCl	0.02% w/v
Na_2HPO_4	0.02% w/v
KH_2PO_4	0.15% w/v

Polymerase Chain Reaction (PCR) and Reverse-transcription (RT-) PCR analysis

10X Tris-borate-EDTA solution	
(made up in dH_2O)	
Tris	10.8% w/v
Boric acid	5.5% w/v
0.5M EDTA pH 8.0	4% w/v

DNA loading buffer

Glycerol	49.9% v/v
10X TBE	49.9% v/v
Bromophenol blue	0.2% w/v

Denaturing Solution

(made up in dH_2O)	
NaCl	1.5M
NaOH	0.5M

Neutralising Solution

(made up in dH_2O)	
NaCl	1.5M
Tris-HCl pH 7.2	0.5M
EDTA pH8.0	0.001M

20X Standard saline citrate (SSC)

(made up in dH_2O)	
NaCl	3M
Na_3 citrate	0.3M

100X Denhardt's Solution

(made up in dH_2O)

BSA	2% w/v
Ficoll	2% w/v
PVP	2% w/v

Formamide

Deionised in 10% mixed bed resin

1X Tris-EDTA buffer

(made up in dH_2O)

Tris-HCl pH 8.0	10mM
EDTA pH8.0	1mM

Pre-Hybridisation solution

(made up in dH_2O)

Formamide	50% v/v
20X SSC	25% v/v
50X Denhardt's solution	10% v/v
SDS	5% w/v
Sheared Salmon sperm DNA	1% v/v

Hybridisation solution

As Pre-hybridisation solution with ^{32}P-labelled oligonucleotide probe and fresh 1% v/v sheared salmon sperm DNA

Post-Hybridisation Wash solutions

2X SSC/ 0.1% w/v SDS

1X SSC/ 0.1% SDS

3.6 Cell Lines

The cells types shown in Table 3-1 were used as experimental controls as described in the appropriate sections:

Table 3-1. Control cell lines

Cell name	Description	Reference
B95-8	EBV transformed marmoset cell line.	Miller *et al* 1972
LCL	Human B-cell line transformed *in vitro* with B95-8	Lam & Crawford 1995
Namalwa	BL tumour derived cell line containing 1-2 copies of EBV genome per cell	Klein & Dombos 1973. Lawrence *et al* 1988
BJAB	EBV negative BL derived cell line	Rowe *et al* 1987
C19	NPC derived tumour material passaged in SCID mice	Busson *et al* 1988
M148	EBV positive BL derived cell line	Rowe *et al* 1987

Cell lines were cultured in a humidified incubator (Leec) at 37°C and at 5% CO_2 with twice weekly feeding with culture medium.

3.6.1 Cell viability assay/ cell counts

Cell viability was assessed using the principal that live cells are able to exclude the dye Trypan blue, whereas dead cells take up the stain. 10µl of 0.5% w/v Trypan blue in PBS solution was mixed with 10µl of cell suspension, and the mixture placed into the chamber of a haemocytometer. Viable cells (unstained) were counted by light microscopy.

3.6.2 Thawing of cell lines and establishment of cultures

Vials of viably frozen cells were thawed in a water bath at 37°C and resuspended by dropwise addition of 1ml of culture medium and transferred to 9ml of culture medium in a universal tube. Cells were centrifuged at 160g for 7 minutes, resuspended at 1 x 10^6 cells/ml in culture medium and transferred to a 25ml tissue culture flask for propagation in a CO_2 incubator as described.

3.6.3 Storage of cell lines

Cells were counted as described using the Trypan blue exclusion method to determine cell viability, aliquoted into freezing medium at 1 x 10^7 cells vial in cryotubes and slowly frozen at -70°C. Vials were transferred to the vapour phase of a liquid nitrogen tank for long-term storage.

3.6.4 Snap freezing of cells

Cells to be used for DNA and RNA extractions were counted and washed in PBS. Aliquots of up to 1 x 10^7 cells were placed in sterile cryovials and centrifuged at 160g for 7mins. The PBS was aspirated off and the vial placed in liquid nitrogen to 'snap-freeze' the cell pellet. The vial was then stored at -70°C overnight and transferred for long term storage in liquid nitrogen.

3.6.5 Cell line dilution series

Ten-fold dilution series of cell lines were established to provide a means of quantifying PCR reaction sensitivity. Dilutions of EBV positive cells were made in a background of negative cells. Thus viable EBV positive and negative cultured cells were counted as described and each diluted to 1 x 10^6 cells/ ml. 9ml of EBV negative cells (9 x 10^6 cells), were aliquoted into universal tubes and 1ml of the EBV positive cells (1 x 10^6 cells) added to the first tube, mixed and 1ml removed and transferred to the next tube, and so on to give a ten fold dilution series of cells from undiluted positive cells to 1 EBV positive cell in a total of 1 x 10^7 cells. The mixtures were centrifuged at 160g for 7 minutes washed in PBS and the cell pellets snap frozen and stored at -70°C for DNA or RNA extraction.

3.6.6 Establishment of tumours in SCID mice

CB17 Severe combined immunodeficient (SCID) mice, maintained under aseptic conditions were injected intraperitoneally with a sterile suspension of cells teased from PTLD biopsy material. On showing signs of being unwell, or after a predetermined time point, animals were culled by cervical dislocation, dissected aseptically and tumour mass and macroscopically affected organs removed. This material was divided into four pieces for processing: a) snap-frozen in a sterile cryovial in liquid nitrogen for subsequent DNA and RNA extraction and PCR analysis, b) Fixed in neutral buffered formalin, or c) snap frozen in OCT for cryostat sections to be cut for histopathological/ immunohistopathological analysis and d) a portion of tumour was aseptically processed to produce a single cell suspension for passage into another SCID mouse. This work was carried out by Dr. Ingolfur Johannessen.

3.7 Clinical samples and controls

3.7.1 Separation of peripheral blood mononuclear cells (PBMCs) from heparinised whole blood

Heparin-treated fresh whole blood (10^3IU/ml) was layered onto an equal volume of Ficoll-Hypaque and centrifuged at 540g for 20 minutes (Boeyum 1968). The upper layer of plasma was harvested and stored at-20°C for serological analysis. The interface, containing PBMCs was harvested and washed twice in wash medium by centrifugation at 160g for 7 minutes. Viable PBMCs were counted by Trypan-blue dye exclusion and aliquoted to be either snap-frozen in liquid nitrogen for DNA/ RNA extraction or frozen viably as described.

3.7.2 Serological analysis

Previous infection with EBV was determined by detection of IgG antibodies against EBV viral capsid antigen (VCA) by indirect immunofluoresence (Henle & Henle 1966). Test slides were prepared by placing 5 x 10^4 washed cells of the EBV containing P3HR1 cell line onto each of 12 wells on a microscope slide. The cells

were allowed to dry at room temperature (RT) and then fixed in acetone for 5 minutes. The slides were air dried at RT and stored at -20°C.

To detect anti-VCA antibodies, slides were thawed at RT and the test sera, diluted in PBS at 1:5 and 1:10 were added to separate spots on the slide alongside known VCA-positive and VCA-negative control sera. The slides were incubated for 1 hour at 37°C in a humidified chamber then washed in PBS at room temperature. 10µl of FITC conjugated rabbit anti human IgG (diluted 1:50 in PBS) was added to each well. Slides were incubated for 1 hour at RT and washed in PBS. PBS:glycerol (1:1) was used to mount coverslips before viewing under an epifluorescent microscope. EBV seronegativity was assumed if staining of the cells was not visible upon repeated testing of the serum at 1:5 dilution.

3.8 Polymerase Chain Reaction (PCR)

PCR amplification was performed to detect EBV and human gene sequences and RNA molecules in nucleic acid extracts. The methods used derive from those described by Saiki *et al* (1985, 1988) and Hart *et al* 1988.

3.8.1 Extraction of Nucleic Acids

3.8.1.1 General Precautions

During extraction of nucleic acid for use in PCR analysis, care was taken to avoid contamination of samples with amplified PCR products which could give rise to false positive results. This was achieved by separating the processing and storage of PCR products from the laboratory where extractions were performed and PCR reactions set up, and using sterile plasticware and pipettors and racks kept exclusively for each procedure.

Additional safeguards were necessary for processing and handling RNA samples due to the risk of contamination with RNAse enzymes from the environment. RNAse-free microfuge tubes and pipette tips containing filters (Anachem) were used for all procedures involving RNA and gloves were changed frequently.

3.8.1.2 Extraction of DNA

Genomic DNA was extracted from stored frozen pellets of cultured cells or PBMCs using a kit ('EZ-DNA' Invitrogen), following the methods described in the manufacturers protocol. Briefly, 1 x 10^6 cells were resuspended in 200µl PBS and 350µl alkaline lysis solution (solution A) added. The mixture was vortexed until the cells were evenly dispersed and incubated at 65°C for 10 mins. 150µl of solution B was added and vortexed until the mixture became uniformly viscous. 500µl of chloroform was added and vortexed until the mixture became homogenous. The mixture was centrifuged for 15 mins at 12 000g to separate the aqueous and organic phases and the upper aqueous phase removed and transferred to a fresh microfuge tube. The DNA was precipitated from solution by addition of 1ml 100% Ethanol which had been pre-chilled to –20°C and incubation on ice for 30 mins. The DNA was separated by centrifugation for 15 mins at 12 000g and the ethanol removed. The DNA pellet was washed in 500µl 80% ethanol, centrifuged for 2 mins, the ethanol removed and the DNA pellet resuspended in 50µl of nuclease free water (Gibco BRL). Contaminating RNA was degraded by treatment with RNAse supplied with the kit (final RNAse concentration (40µg/ ml). DNA concentration was determined by measuring the samples absorbance at 260nm and 280nm in a Perkin Elmer DNA/ RNA calculator and all samples were diluted to 100ng/µl and stored at -20°C for subsequent use. DNA samples extracted in this way had a 260/ 280 ratio between 1.8-1.9.

3.8.1.3 Extraction of RNA

Total cellular RNA was extracted by homogenisation of frozen cell pellets by pipetting several times in RNAzol B solution (Cinna/ Biotecx) (2ml/ 10^7 cells). Frozen tissue samples were homogenized in the solution using sterile RNAse free homogenizer rods. The manufacturers protocol was followed. Briefly, following complete disruption of the cells or tissue in RNAzol B, 100µl of chloroform was added per 1ml of homogenate and the samples were then tightly capped and shaken vigorously for 15 seconds and kept on ice for 5 mins. The mixture was then

centrifuged in a microfuge at 12 000g for 15 mins at +4°C to separate the aqueous and organic phases and the upper aqueous phase removed to a fresh RNAse-free microfuge tube. RNA was precipitated by addition of an equal volume of isopropanol and storage at +4°C for 15 mins. The RNA was pelleted by centrifugation at 12000g for 15mins and the supernatant removed. The RNA pellet was washed once in 800µl of 75% ethanol and centrifuged for 8 mins at 7 500g. The supernatant was removed and the RNA pellet resuspended in 50µl nuclease free water (Gibco BRL). RNA concentration was determined by spectrophotometry as above and the sample diluted to 100ng/µl. To avoid degradation by repeated freeze/ thaws or contamination, all samples were separated into two aliquots for storage at -70°C and one aliquot was used for subsequent studies with the second as back up.

3.8.1.4 Complementary DNA (cDNA) synthesis

cDNA was synthesized by reverse transcription of 1ug of total RNA using Moloney Murine Leukaemia virus (MMLV)-reverse transcriptase (RT) and random hexamer primers (Pro-star RT-PCR kit, Stratagene). The reaction was set up as shown below, and incubated at 37°C for one hour following which cDNA preparations were used immediately or stored at -20°C for subsequent use.

Sample total RNA	1µg
Random primers	0.3µg
First-strand buffer	1X
RNAse inhibitor	40U
deoxynucleotide-triphosphates (dNTPs)	25mM per dNTP
MMLV-RT	50U
dH_20	to 50µl total volume

3.8.1.5 PCR Primers

Oligonucleotide primer sequences were either obtained from published sources, or were designed for this study. All primers and probes are described in

Table 3-2. New primers were designed ensuring there was no sequence complementarity within or between the primer pairs to avoid self-annealing. Validation and optimisation experiments were performed as described in this section to ensure that the correct sequences were amplified with the greatest possible sensitivity and specificity. Oligonucleotides were synthesised by Gibco BRL and diluted to 100ng/µl for storage in aliquots at -20°C to avoid degradation or contamination.

3.8.1.6 Optimisation of PCR Reaction conditions

Optimisation experiments were performed to ensure the reaction conditions gave maximum efficiency and specificity to the amplification. The most important factors tested were $MgCl_2$ concentration and annealing temperature. The starting point for optimising annealing temperature was to calculate the melting temperature through an equation based on the relative amounts of GC and AT nucleotides in each primer:

Melting Temperature (Tm°C) = 4 x (GC) + 2 x (AT) - 5

Initial PCR reactions were carried out on replicates of appropriate control material under standardised reaction conditions (ie 92°C 1 min, mean annealing temperature of the primer pair (Tm°C) 1 min, 72°C 1min for 30 cycles), with titration of $MgCl_2$ concentrations. The $MgCl_2$ concentrations tested were 1mM, 1.5mM, 2mM, 2.5mM and 3mM. The optimal $MgCl_2$ concentration was determined subjectively by viewing ethidium bromide (EtBr) stained gels of PCR products and autoradiographs. That which gave the best combination of high sensitivity (bands visible at greater dilutions of positive cells) and low levels of non-specific bands was selected.

Using the optimal $MgCl_2$ concentration determined from this experiment, the PCR annealing temperature was optimised. Four identical sets of reactions were set up to amplify sequence from the control material with the same reaction mixture containing the optimised $MgCl_2$ concentration. The four sets of reactions were simultaneously placed in thermal cyclers under identical conditions other than

annealing temperature. The annealing temperatures tested were based upon the melting temperature of the oligonucleotides: Tm, Tm-5, Tm +5, Tm-10°C. The criteria for determining the optimal annealing temperature were a combination of reaction sensitivity and specificity as described.

Table 3-2. Oligonucleotide Primers

Target sequence	Oligonucleotide sequence (5'-3') (Published sources shown overleaf)	EBV genome coordinates	Product size
Human β-actin 5'	GTGGGGCGCCCCAGGCACAC	-	
Human β-actin 3'	CTCCTTAATGTCACGCACGATTTC	-	
Human β-actin probe	GGATAGCAACGTACATGGCT		450bp
EBER1 5'	AAAACATGCGGACCACCAGC[3]	6629-6648	
EBER1 3'	AGGACCTACGCTGCCCTAGA[3]	6795-6777	
EBER1 probe	ACGGTGTCTGTGGTTGTCTT[3]	6718-6737	166bp
EBNA3c 5'	GGCTGTCAAGAATCGCACCT	98721-98815	
EBNA3c 3'	GTGTTTAGAGTTCGTGCCGC	99107-99087	
EBNA3c 5' nested	CATCTTGTGCTTCGTGATGG	98864-98883	
EBNA3c 3' nested	TAACATGATGCTGTCAGCCC	99062-99043	
EBNA3c probe	GGCGACATTGGCTTCTAACA	98947-98966	198bp
LMP1 5'	ACACACTGCCCTGAGGATGG[1]	169490-169470	
LMP1 3'	ATACCTAAGA/CAAGTAAGCA[1]	168956-65/9042-51	
LMP1 5' nested	ACAATGCCTGTCCGTGCAAA	169081-169100	
LMP1 3' nested	CTTCAGAAGAGACCTTCTCT	169262-169243	
LMP1 probe	CTACTGATGATCACCCTCCT[1]	169216-169119	182bp
LMP2A 5'	ATGATCCATCTCAACACATA[1]	166874-166893	
LMP2B 5'	CAGTGTAATCTGCACAAAGA[1]	169819-169838	
LMP2A/B 3'	TCACCAGAACGTAAATGCCT[1]	361-380	
LMP2A/B 5' nested	CTCGTGTTTCACGGCCTCAG	124-143	
LMP2A/B 3' nested	AAGGTGGGTCCTCAATCCTC	401-382	
LMP2A/B probe	CTCTCACTTCTACTCTTGGC	176-200	260bp
Gp350 5'	CACAGGCCCCACTGTATC[2]	2402-2422	
Gp350 3'	GAGGTGGAGCTGGTCATTG[2]	2544-2524	
Gp350 probe	AGATGGACTTGGTGTCAC[2]	2460-2480	142bp

Oligonucleotide primer sequences from previously published sources: 1. Brooks *et al* 1992. 2. Rochford & Mosier 1995. 3. Tierney et al 1994. All other oligonucleotide sequences were designed for this study.

3.8.2 Gel electrophoresis of PCR products

PCR products were resolved and visualised by gel electrophoresis according to the protocols of Sambrook, Fritsch and Maniatis (1989). A 2.5% agarose gel was made by melting Low viscosity NuSieve 3:1 agarose (3 parts agarose, 1 part SeaKem agarose) by microwaving in 1X TBE buffer. Following cooling the dissolved agarose to 'hand-hot' temperature by holding the flask under running water, EtBr (10 μg/ml) was added to 1μg/ml. The agarose was poured into a gel casting tray with combs inserted and allowed to set. The gels were placed in the electrophoresis tank (Scotlab), covered with 1x TBE buffer, and the combs carefully removed.

To load the PCR products, 25μl (25% of the total volume) was mixed with 5μl of 6X loading buffer and carefully pipetted into the appropriate wells. Two sets of DNA size markers were loaded on each gel: ^{32}P labelled HinfI-digested ϕX174 DNA and non-radiolabelled 1Kbp DNA ladder (Gibco BRL). Electrophoresis was carried out at 80mA and EtBr stained DNA was visualised under a UV transilluminator gel documentation system (UVP) and a photograph taken for record.

3.8.3 Southern Transfer of PCR products

Following electrophoresis of PCR products, the DNA was transferred onto Nylon membrane according to the method of Southern (1975). The gel was first rinsed in dH20 and soaked in an alkaline DNA denaturing solution for 30 minutes and then transferred to a pH 7.2 neutralising solution for 30 minutes. To transfer the DNA from the gel to a nylon membrane, the gel was placed on top of a strip of 3MM blotting paper laid across a glass plate supported over a tray of 10X SSC solution. The 3MM paper had been soaked in 10X SSC before being laid over the plate, and both ends of the paper hung over the edges into the solution to act as a wick. The area

around the gel was surrounded closely with cling film to prevent the subsequent layers from getting wet from the surrounding paper. Three pieces of 3MM paper and one piece of positively charged nylon membrane (Hybond N+) were cut to the exact size of the gel, and layered on top of the gel: firstly the membrane followed by a 10X SSC wetted piece of 3MM paper, and the remaining pieces of paper. Care was taken to exclude air bubbles from between the layers. Finally a stack of dry paper towels was placed on top of the paper followed by a glass plate and a 500g weight. This arrangement causes 10X SSC solution to be wicked upwards through the gel, transferring the DNA from the gel onto the nylon membrane. Following transfer at room temperature overnight, the apparatus was dismantled and the membrane marked to indicate orientation. The membrane was then rinsed briefly in 2X SSC, dried on 3MM paper and then the DNA was fixed onto the membrane by cross-linking in a Stratalinker™ at 120, 000 μJ UV.

3.8.4 Hybridization of Membranes

PCR products were detected by Southern hybridisation of the DNA fixed onto nylon membranes using a ^{32}P labelled oligonucleodide probe.

3.8.4.1 Pre-Hybridization

To reduce non-specific binding of labelled probe, the membranes were blocked by incubation in a Hybaid hybridisation oven for two hours at 42°C in pre-hybridization solution containing 1% v/v denatured and cooled sheared salmon sperm DNA.

3.8.4.2 ^{32}P End labelling of Oligonucleotide probes

Specific oligonucleotide probes were designed and are detailed in the Table 3-2. Working solutions of probes were diluted to 10μM and stored at -20°C.

Oligonucleotides were 5' end labelled with ^{32}P γATP by T4 polynucleotide kinase (T4 PNK) (Promega) incubated for 30 minutes at 37°C. The reaction contents are:

Oligonucleotide	10pMoles
10X Buffer	2μl
^{32}P γATP	1.85MBq
T4 PNK	1unit
dH_2O	to 20μl final volume

The labelled oligonucleotide probe was separated from unincorporated ^{32}P γATP by running the mixture through a pre-wet column containing Sephadex G50 sepharose (NICK™ column, Pharmacia). Purified radiolabelled probe was eluted from the column in 1X TE.

3.8.4.3 Hybridisation of blots

Following pre-hybridization, the ^{32}P-labelled oligonucleotide probe was added to 25ml of Pre-hybridization solution containing freshly denatured and cooled sheared salmon sperm DNA (1% v/v). The pre-hybridization solution was removed from the blots and replaced with probe solution. Hybridization of the labelled oligonucleotide to the membrane-immobilized PCR products was carried out by incubation overnight at 42°C.

3.8.4.4 Autoradiography

Following hybridization, probe solution was removed and stored at -20°C. Blots were washed to remove non-specifically bound probe from the membrane. Following each wash the blots were checked using a Geiger counter, and increasingly stringent washes were used to reduce background levels of radioactivity on the blot. Initial washes were twice for 10 minutes in 2X SSC, 0.1% w/v SDS followed by 1X

SSC, 0.1% SDS and if necessary 0.1X SSC, 0.1% SDS. Washed membranes were removed from the solution and drained of excess moisture on 3MM paper. Blots were then covered in cling film and exposed to X-ray film in autoradiography cassettes with an intensifying screen for 1 to 7 days at -70°C. Developed autoradiographs were labeled and stored.

Radiolabelled DNA size markers were visible on autoradiographs for size determination of bands visible through hybridization of labelled probe to the complementary sequence of the PCR product. PCR reactions were repeated if bands were visible in water or negative control lanes indicating contamination of the reaction or if no bands were visible in the positive controls, indicating that the reaction or hybridization had failed. A band of correct size in a sample lane was considered positive, and if no band for a particular sample was visible following exposure of the blot for 7 days, the sample was considered negative.

3.8.5 EBV Semi-quantitative PCR

In order to evaluate the EBV copy number in the PBMs of patient samples, a semi-quantitative PCR method was established. PCR was carried out on a Namalwa dilution series containing 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10 and 1 EBV genome copies/ 10^7 cells (Lawrence *et al* 1988). DNA was extracted from the cell mixtures and PCR performed to amplify the highly conserved Bam H1 W repeat region of the EBV genome (Wagner *et al* 1992). Following electrophoresis and Southern blotting of the PCR products, autoradiographs were analysed by scanning densitometry (Phoretix 1D Advanced package) to determine the volume of the amplified bands. In addition to optimization of $MgCl_2$ concentration and annealing temperature, the number of amplification cycles was optimised such that amplification of the Bam H1 W PCR product was in the linear part of the amplification profile. This was done to ensure a linear relationship between amplified band volume and the starting template copy number. PCRs were performed over 20 to 30 cycles and the band volume plotted against cycle number to determine the optimal cycle number which gave maximum product yield in the linear portion of the graph (shown in Results Figure 4-7).

DNA was extracted from clinical samples and controls as described, and 1ug DNA used as template in all reactions. Alongside each set of clinical samples, Bam H1 W PCR was performed on the complete Namalwa dilution series. In addition a PCR for detection of the human β–globin gene was performed on all samples. Since 1ug DNA was used for each reaction equivalent amounts of PCR product should be detected for all samples, and no amplification or low product yield would indicate that the DNA concentration was incorrect or the sample contained inhibitors of the PCR reaction. This therefore acted as a control for differences between samples to avoid misleading results. Band volumes were determined by scanning densitometry of Bam H1 W and β–globin autoradiographs, and Bam H1 W band volume figures were adjusted on the basis of the β–globin PCR through comparison with the BJAB as standard control. Band volumes for Namalwa dilution series were plotted against EBV copy number allowing EBV genome copy number in clinical samples to be determined through reading band volume from the graph.

4 Results

Studies were carried out to determine changes in EBV genome copy number and EBV gene expression in patients undergoing cardiothoracic transplantation in order to establish the influence of iatrogenic immunosuppression on EBV infection *in vivo* and how this may relate to development of PTLD.

Blood samples were taken from patients immediately prior to and at intervals following transplantation with follow up times of up to three years. Observed changes in EBV gene expression and genome copy number were studied in comparison to latent infection in normal healthy carriers, individuals undergoing symptomatic primary infection (IM) and patients with EBV associated PTLD.

4.1 EBV Semi-quantitative PCR

A semi-quantitative PCR was developed to determine EBV copy number in the peripheral blood of transplant recipients and assess how changes in EBV load relate to immunosuppressive therapy and clinical status.

The primers and conditions for the Bam H1 W and β-Globin PCRs have been published previously (Wagner *et al* 1988, Saiki *et al* 1985). However it was important to optimise PCR reaction conditions.

4.1.1 Optimisation of Bam W and β-Globin PCR conditions

The reaction conditions of each PCR were standardised to ensure optimal sensitivity and specificity of detection. It was also necessary to demonstrate that product band intensity was proportional to starting target concentration to provide a semi-quantitative assessment of EBV load in the samples tested. This was achieved through determining the cycle number which gave maximum product in a linear relationship to the target concentration.

4.1.1.1 Optimisation of $MgCl_2$ concentration

$MgCl_2$ is required for enzymatic function of Taq polymerase and varying its concentration alters product yield (Linz *et al* 1990).

1μg of DNA from a Namalwa dilution series (Materials and Methods section 3.1.5) was used in PCR reactions to determine the optimal $MgCl_2$ concentration as described in Materials and Methods Section 3.3.1.6. Four $MgCl_2$ concentrations were tested; 1mM, 1.5mM, 2mM and 2.5mM under identical reaction conditions. For optimisation of Bam H1 W PCR $MgCl_2$ concentration, each Namalwa dilution was tested at each $MgCl_2$ concentration to establish how $MgCl_2$ concentration affected reaction sensitivity (Figure 4-1a and b). For the β-Globin PCR, DNA from a single Namalwa dilution (1000 genomes/10^6 genomes) was used in triplicate at each $MgCl_2$ concentration (Figure 4-2). The optimal $MgCl_2$ concentration for Bam H1 W PCR was taken to be that which gave the clearest most intense band in autoradiographs and detected the lowest concentration of EBV. A graph of Bam H1 W PCR autoradiograph band volume against EBV genome copy number with each $MgCl_2$ concentration is presented in Figure 4-3. A bar chart showing mean autoradiograph band volume from replicates of β-globin PCRs at each $MgCl_2$ concentration is shown in Figure 4-4. From these graphs the optimal $MgCl_2$ concentration for Bam H1 W and β-Globin PCRs were 1.5mM and 1mM respectively.

Figure 4-1 a & b. Bam H1 W PCR $MgCl_2$ optimisation

Figures show autoradiographs of experiments to determine the optimal $MgCl_2$ concentration for Bam H1 W PCR. Samples from a Namalwa cell dilution series containing 1 x 10^7 to 1 EBV genome in 1 x 10^7 cells was tested at $MgCl_2$ concentrations of 1mM, 1.5mM, 2mM and 2.5mM (Materials and Methods section 3.3.1.6). From this experiment, the optimal $MgCl_2$ concentration was 1.5mM (lane B at each Namalwa concentration).

Lane annotation: M: ϕX174 DNA digested with HinfI. Fragment sizes: 726/ 713, 553, 500, 427/ 417/ 413, 311, 249, 200, 151, 140, 118, 100, 82.

dH_20: control for contamination. Figures show EBV genome concentration, ranging from 10^6 genomes/ 10^7 cells to 1 in 10^7. Each dilution was amplified under varying MgCl2 concentrations as shown: **A**: 1mM $MgCl_2$, **B**: 1.5mM $MgCl_2$, **C**: 2mM $MgCl_2$, **D**: 2.5mM $MgCl_2$.

Figure 4-1a. Namalwa concentrations from 10^6 EBV genomes/10^7 cells to 10^4 genomes/10^7 cells

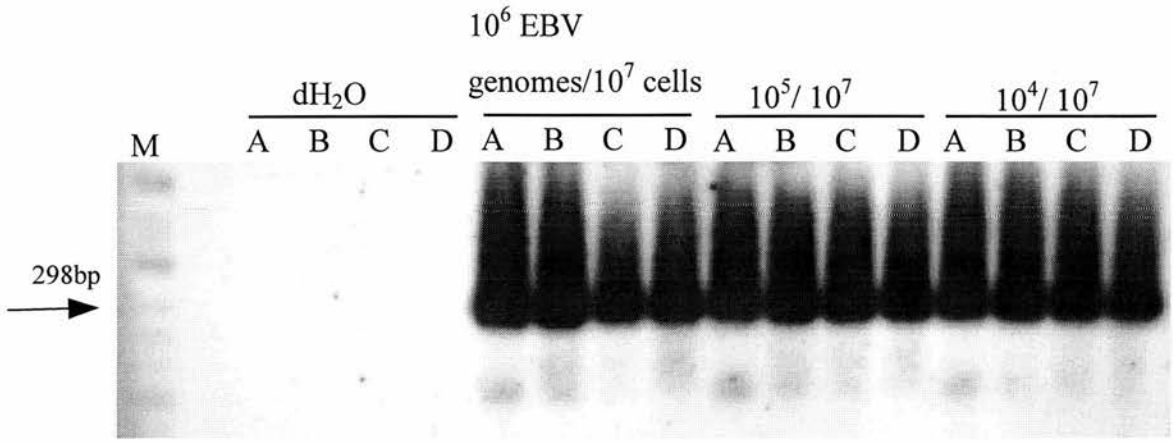


Figure 4-1b. Namalwa dilutions from 10^3 EBV genomes/10^7 cells to 1 EBV genome /10^7 cells. (Annotation as Figure 4-1a).

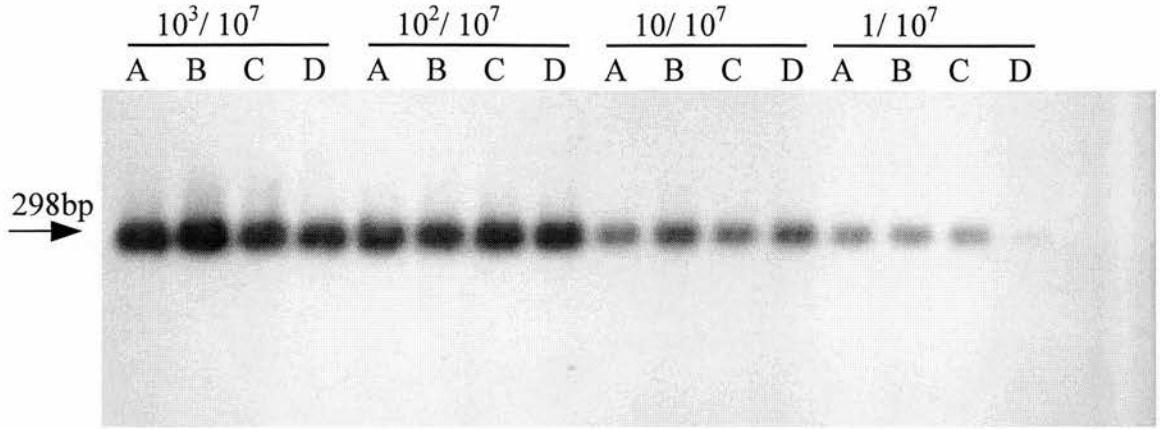


Figure 4-2. β-globin PCR $MgCl_2$ optimisation

Three replicate β-Globin PCRs (Replicate 1, 2 and 3) were carried out on 1μg DNA from a single Namalwa dilution (10^3 EBV genomes/ 10^7 cells) at each $MgCl_2$ concentration.

Lane annotation: M: Markers. ϕX174 DNA digested with HinfI. **dH_20**: Control for contamination. **BJAB**: EBV negative cell control. **A**: 1mM. **B**: 1.5mM. **C**: 2mM. **D**: 2.5mM $MgCl_2$

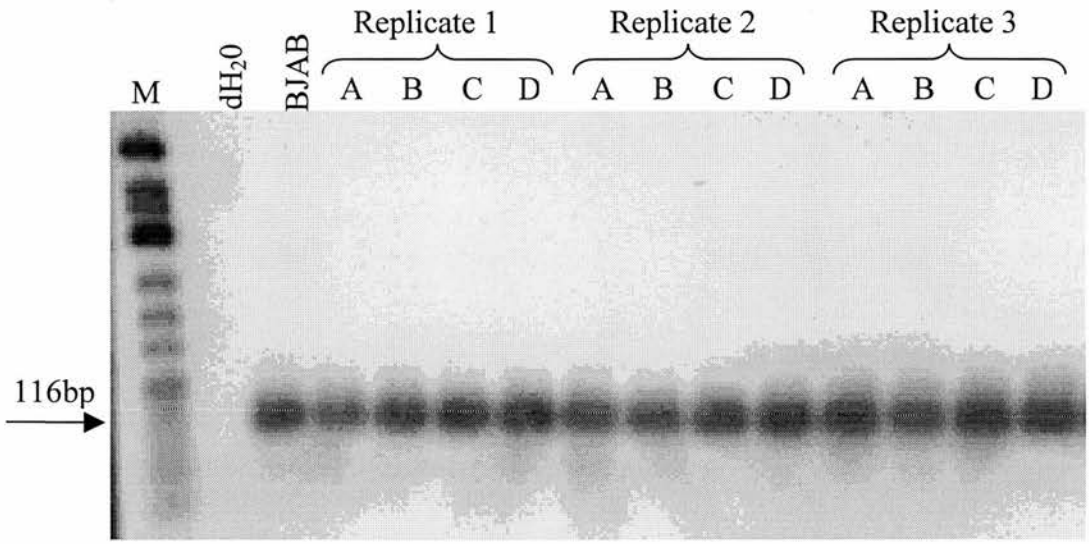


Figure 4-3. Graph of Bam W $MgCl_2$ PCR optimisation data

Graphs shows autoradiograph band volumes from Figures 4-1 and b plotted against EBV genome copy number at each $MgCl_2$ concentration tested. From this graph, the $MgCl_2$ concentration giving the most direct correlation between EBV genome copy number and band volume is 1.5mM (green line).

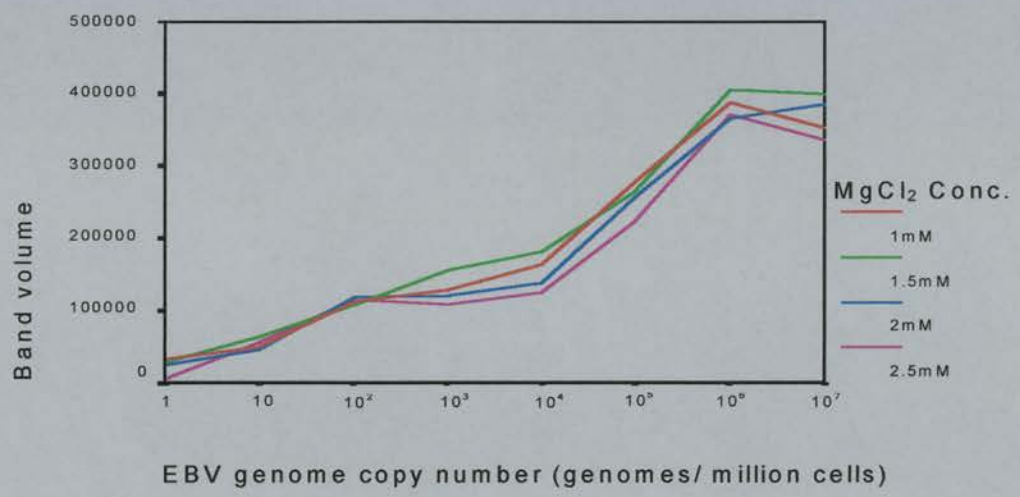
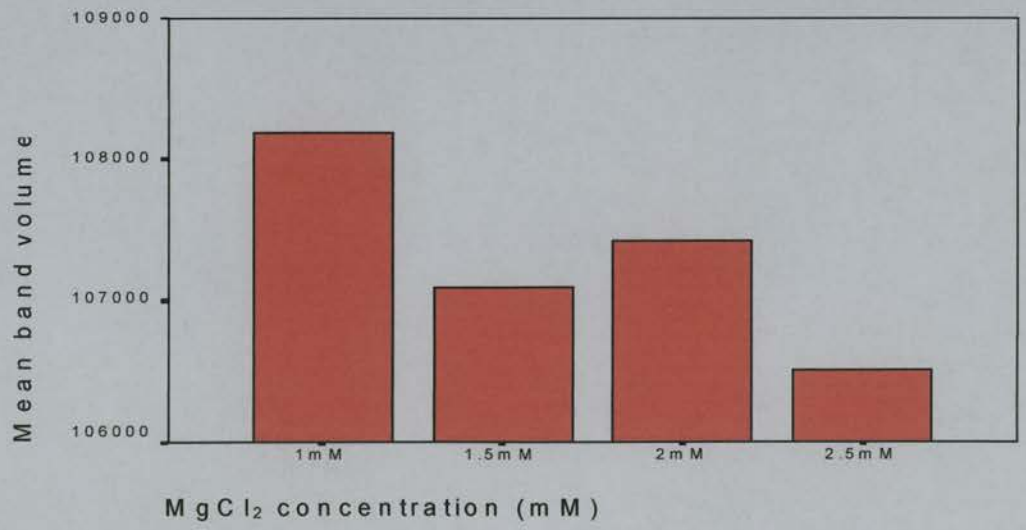


Figure 4-4. Bar chart of β-globin $MgCl_2$ optimisation data

Chart shows the mean value from three replicates of each $MgCl_2$ concentration. The selected concentration was 1mM.



4.1.1.2 Optimisation of annealing temperature

Primer annealing temperature is important in determining amplification efficiency and specificity (Kocher & Wilson 1991). 1μg DNA was amplified in a 35-cycle reaction at the optimal $MgCl_2$ concentration determined from the previous experiments. Three different annealing temperatures were used, derived from the calculated oligonucleotide melting temperature (Tm) (Materials and Methods section 3.3.1.6): Tm °C, Tm -5°C and Tm -10°C. The optimal annealing temperature was determined by studying autoradiographs of PCR products and selecting the temperature which gave the clearest, most intense bands and detection of the lowest EBV genome concentration. Autoradiographs from annealing temperature optimisation experiments are shown in Figure 4-5. Graphs showing scanning densitometry band volumes from these blots are shown in Figure 4-6. From these experiments the optimal annealing temperature was 57°C for Bam W PCR and 54°C for β-Globin PCR.

Figure 4-5. Bam W PCR annealing temperature optimisation

Bam H1 W PCRs were performed on DNA from a Namalwa dilution series under reaction conditions identical in all but annealing temperature. Three annealing temperatures were used on DNA from each dilution, LCL and water controls as indicated: A: 62°C, B: 57°C, C: 54°C. M: ϕX174 HinfI

Figure 4-5a. Bam W PCR annealing temperature optimisation. Namalwa dilutions 10^7 EBV genomes/ 10^7 cells to 10^4 genomes/ 10^7 cells

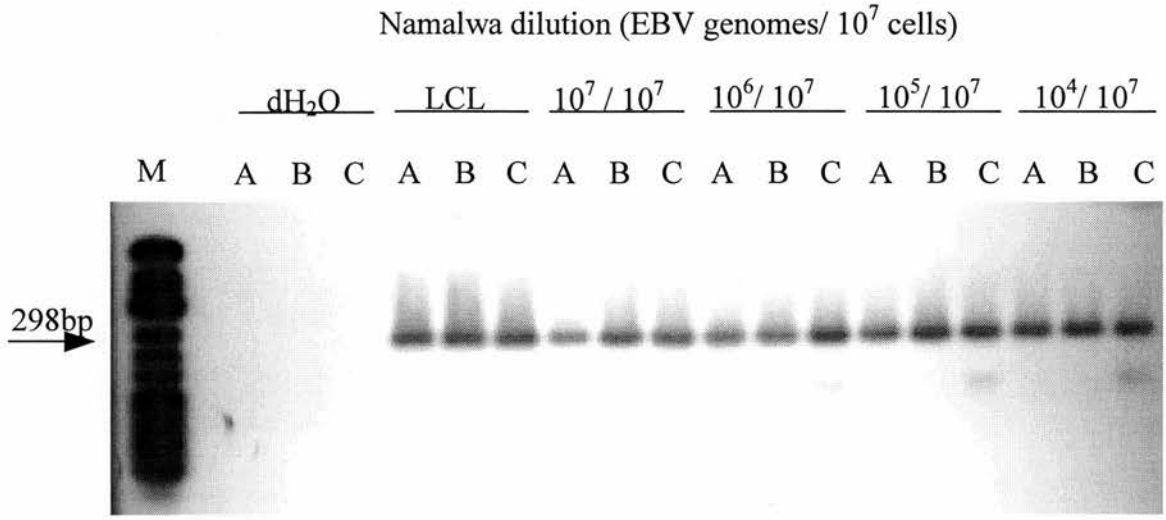


Figure 4-5b. Bam H1 W PCR annealing temperature optimisation.

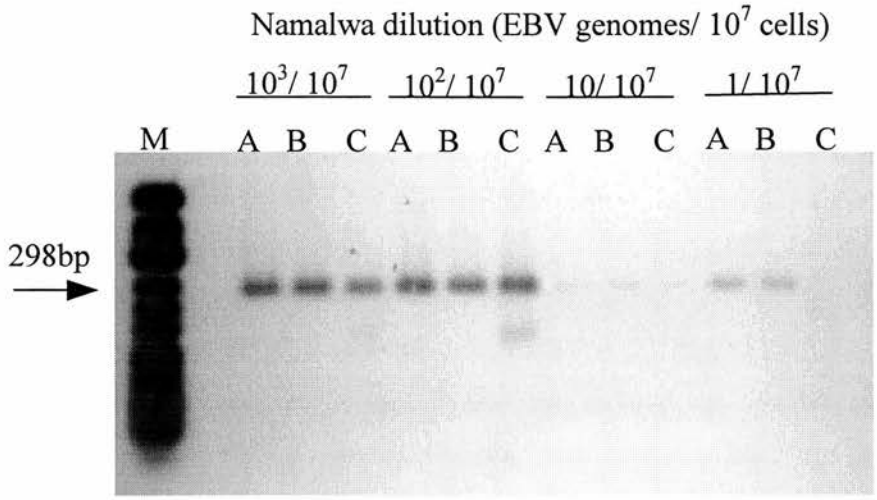


Figure 4-6. Graph of Bam H1 W PCR annealing temperature optimisation

Graph of autoradiograph band volume plotted against EBV genome copy number at three annealing temperatures. An annealing temperature of 57°C (green line on graph) was selected because this gave the best sigmoid curve of the three tested temperatures and greatest sensitivity, yet produced no non-specific bands unlike when using 52°C (Figure 4-5).

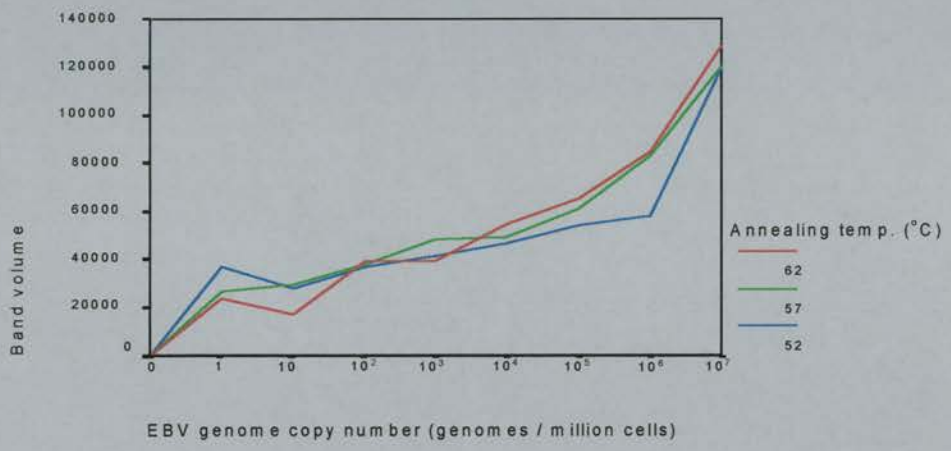
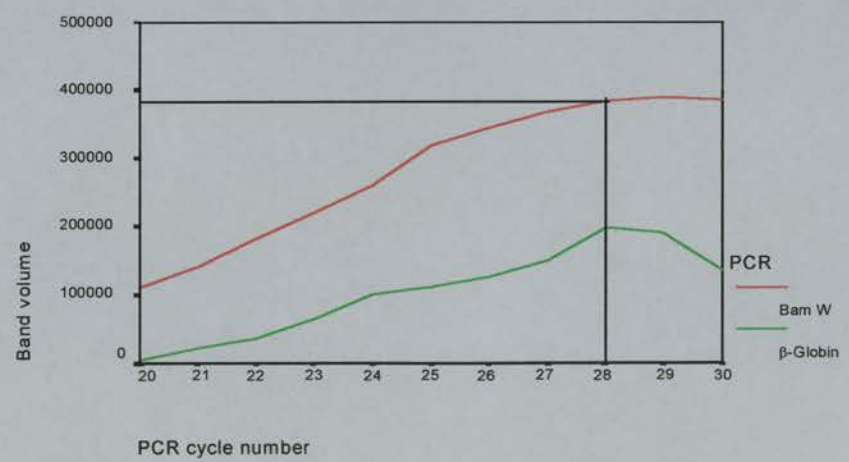


Figure 4-7. Graph of autoradiograph band volume with differing PCR cycle number



28 cycles was chosen as optimal for both Bam W and β-globin PCRs as it gave the highest product yield within the exponential phase of the reaction profile (indicated on the graph by the black line).

4.1.1.3 Optimisation of PCR cycle number

The reaction kinetics of PCR follow a sigmoid curve whereby the amount of product increases exponentially with each cycle until the depletion of reagents or accumulation of inhibitors leads to tailing off of the reaction profile. For PCR to be quantitative the concentration of measurable product must be proportional to the concentration of target molecule at the beginning of the reaction. Thus the reaction must be halted during the exponential phase of the reaction when production of amplimers is proportional to the amount of starting material. Since sensitivity of the reaction is also important it is necessary to select the highest cycle number which remains in the exponential phase of the reaction to give maximum product yield while retaining proportionality to target concentration.

Bam W and β-globin PCR reactions were performed under optimised reaction conditions on eleven identical samples containing 1μg DNA from a dilution of Namalwa cells in a background of EBV negative cells (1×10^3 EBV genomes/10^6 cells) since this dilution gave intermediate band intensity. A sample was removed from the PCR machine after each complete PCR cycle between 20 and 30 cycles. Following electrophoresis, autoradiography (Figure 4-7a and b for Bam W and β-globin PCR respectively) and scanning densitometry, band intensity was plotted against cycle number (Figure 4-8a and b). For both Bam W and β-Globin PCRs, 28 cycles gave maximum band intensity in the exponential phase of amplification and was therefore used in all subsequent experiments.

Figure 4-8. Bam H1W and β-Globin PCR cycle number optimisation

Figure 4-8a. Bam W PCR cycle number optimisation

Autoradiographs of Bam H1 W PCR on Namalwa dilution DNA (10^3 EBV genomes/ 10^7 cells) amplified for between 20 and 30 cycles as indicated.

Controls: M: ϕX174 HinfI. **1. dH_2O:** Water contamination control. **2.** BJAB: EBV negative BL cell line (Negative control). **3.** LCL: Lymphoblastoid cell line (Positive control)

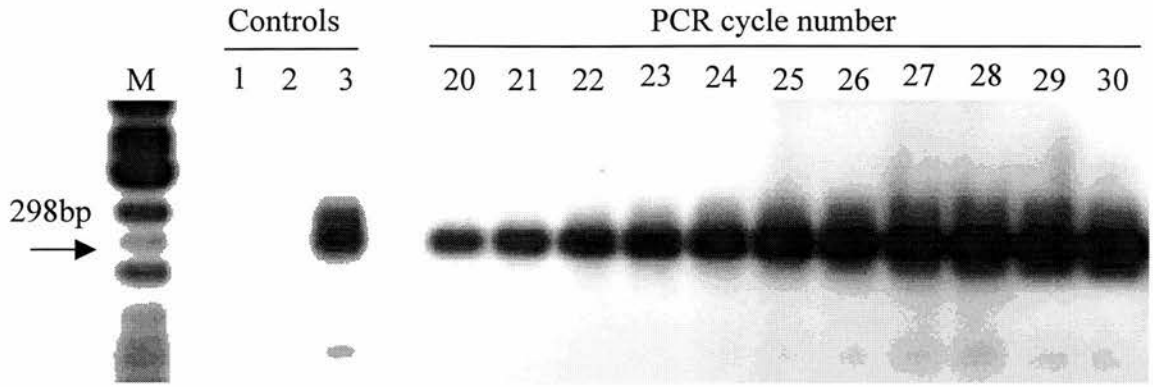
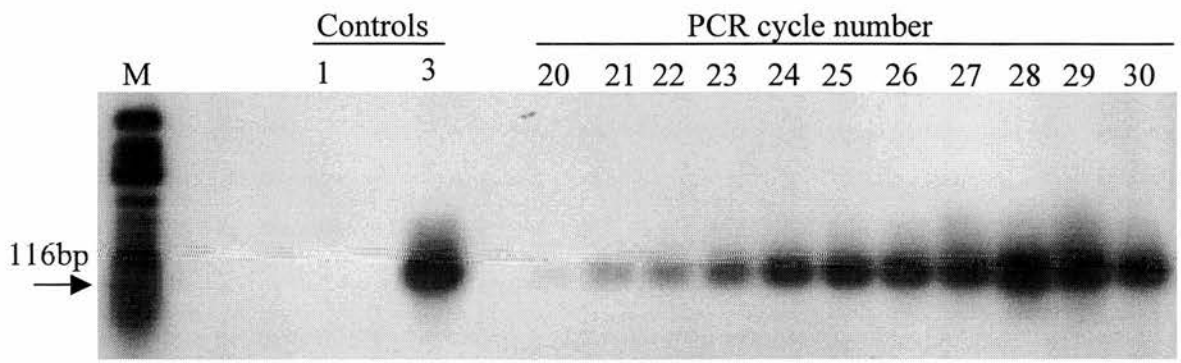


Figure 4-8b. β-Globin PCR cycle number optimisation

Lane annotation as for Figure 4-6a.



4.1.1.4 Effect of DNA concentration on Semi-quantitative PCR

To ensure the accuracy of semi-quantitative PCR it is important to demonstrate that variation in target DNA concentration is reflected in the amount of detectable amplified product. Differences in the amount of target DNA incorporated in PCR reactions may be caused by errors in spectrophotometry, DNA dilution or inaccuracy in aliquoting samples for PCR. An experiment to investigate the correlation between the quantity of DNA incorporated in Bam H1 W and β-Globin PCRs was carried out using Namalwa DNA which had been serially diluted two-fold in sterile water. Autoradiographs of Southern blots obtained from these experiments are shown in Figures 4-9a and b and graphs of autoradiograph band volume plotted against DNA concentration from Bam W and β-Globin PCRs are shown in Figures 4-10 and 4-11 respectively.

The results of this experiment show that autoradiograph band volume using both Bam W and β-Globin PCRs increases in direct proportion to the amount of template DNA included in the reaction. β-globin PCR can therefore be used to estimate the amount of template DNA added at the start of a PCR reaction to allow adjustment of Bam W band volume for differences in DNA concentration between samples.

Figure 4-9. Effect of DNA concentration on Bam H1 W and β-Globin PCR

Autoradiographs of Southern blots generated following Bam H1 W and β-Globin PCR on a two-fold dilution series in dH_2O made from Namalwa DNA (10^3 EBV genomes/ 10^7 PBMs). The amount of DNA in each dilution was 1μg, 0.5 μg, 0.25μg, 0.125μg, 0.06μg and 0.03μg.
M: Marker: ϕX 174 Hinf1 digested. **dH_20**: water control. **BJAB**: EBV negative cell control. **B95-8**: EBV positive cell control

Figure 4-9a. Bam H1 W PCR

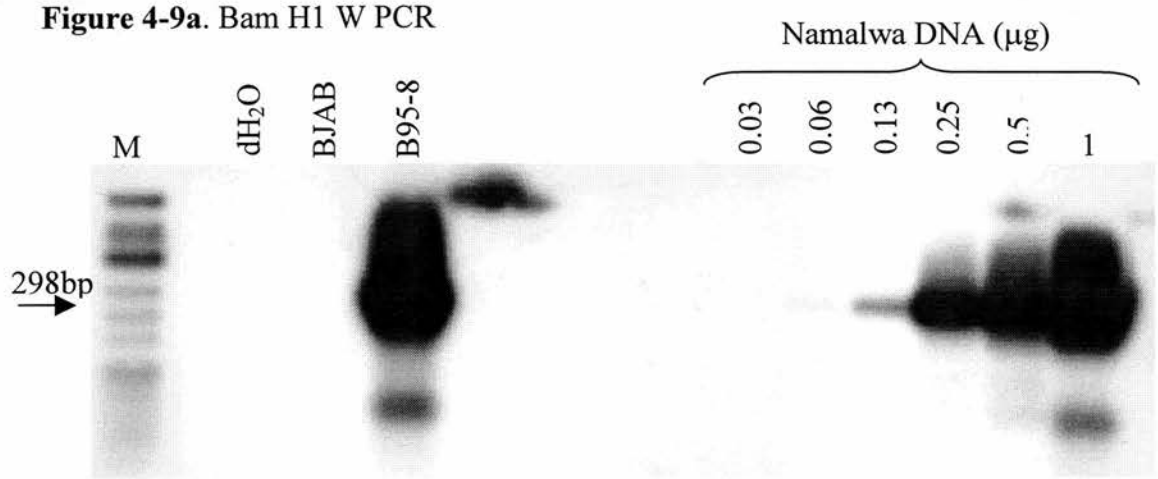


Figure 4-9b. β-Globin PCR

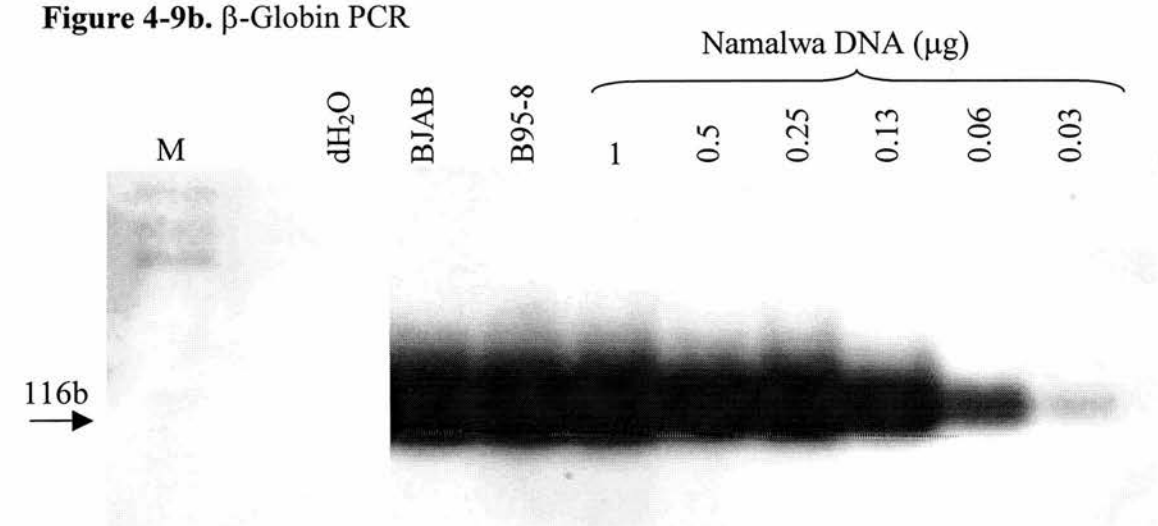


Figure 4-10. Graph showing effect of DNA quantity on Bam H1 W PCR

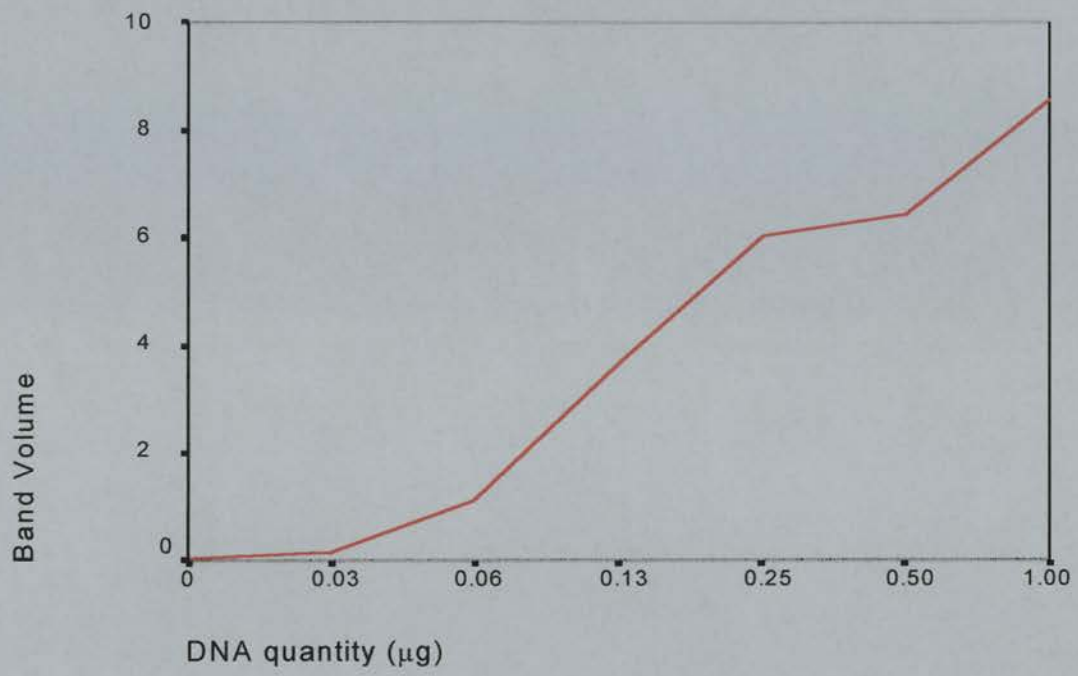
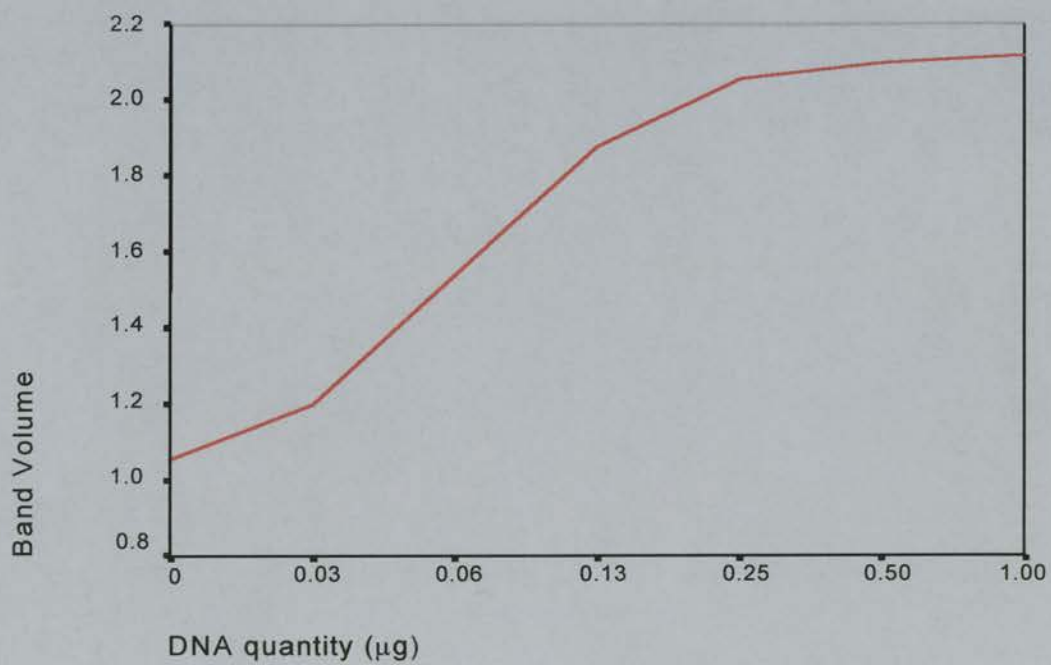


Figure 4-11. Graph showing effect of DNA quantity on β-Globin PCR



4.1.2 EBV genome copy number in control and clinical samples:

4.1.2.1 Normal Healthy individuals

A control PCR was performed alongside clinical samples to provide a standard curve of EBV genome copy number against autoradiograph band volume, which detected 1 EBV genome/10^6 PBMs (Figure 4-12). EBV semi-quantitative PCR was performed on PBMs from thirty-six healthy EBV seropositive and three seronegative adults. Representative autoradiographs of Bam W and β-globin PCRs are shown in Figures 4-13a and b respectively and results for all normal healthy individuals are presented in Table 4-1.

EBV DNA was detected in PBMs from 20 of 36 EBV seropositives (55.6%) with EBV loads ranging from 1 to 200 EBV genomes/ 10^6 PBMs. The 16 samples in which EBV was not detected (44.4%) were described as containing less than 1 copy of the EBV genome per 10^6 PBMs, below the limit of sensitivity of the PCR used. The overall median of EBV copy number in EBV seropositive individuals is 1 EBV genome/10^6 PBMs. The interquartile range of EBV load was calculated as 1-40 EBV genomes/10^6 PBMs, providing a measure of the central range of EBV load between 25% and 75% of the maximum. No EBV DNA was detected in the three EBV seronegative samples tested.

Figure 4-12a-c. Semi-quantitative EBV DNA PCR on Namalwa dilution series

Figure 4-12a. Bam W PCR on Namalwa dilution series

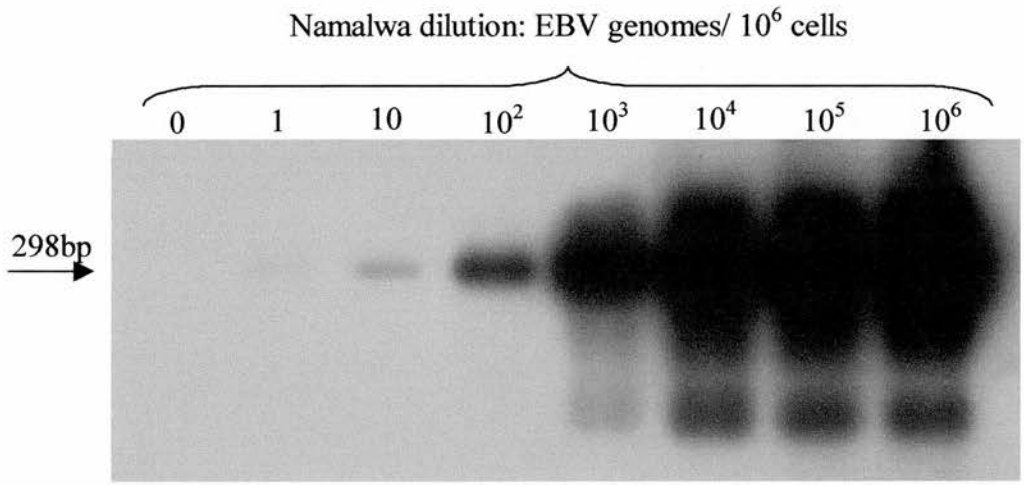


Figure 4-12b. β-Globin PCR on Namalwa dilution series

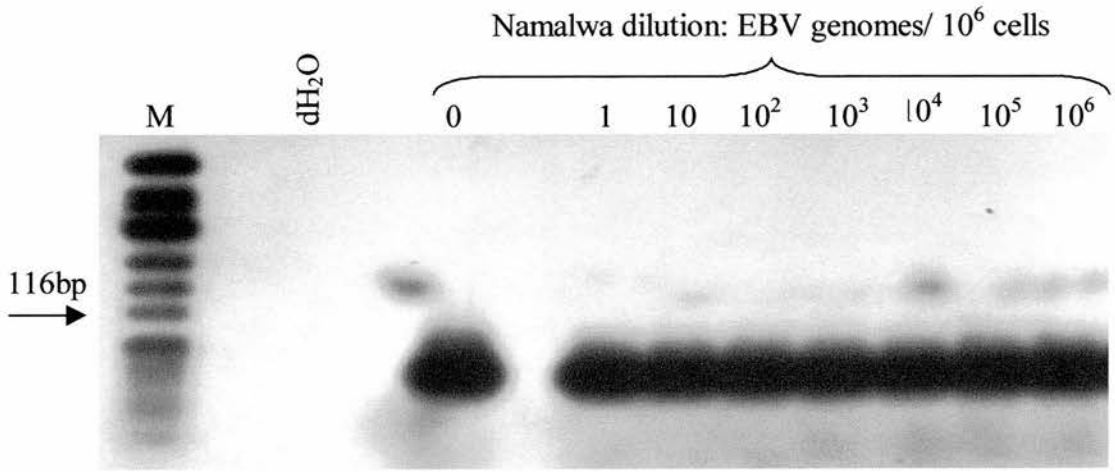


Figure 4-12c. Standard curve of Bam W autoradiograph band volume with Namalwa dilution

EBV copy number was plotted against autoradiograph band volume for Bam W PCR on Namalwa dilution series. This standard curve was used for calculation of EBV genome copy number in clinical samples.

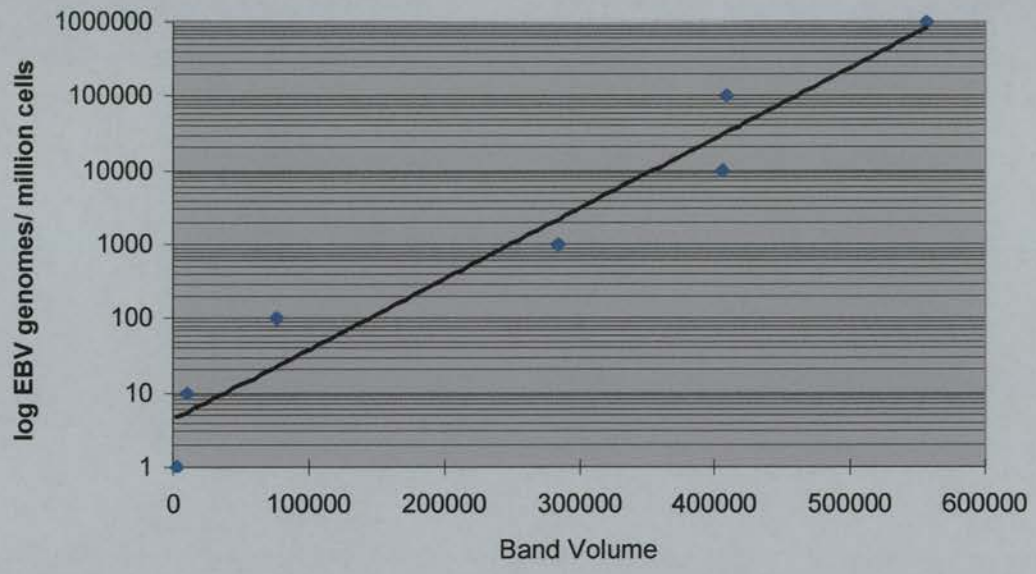


Figure 4-13a & b . Bam W & β-Globin PCR on PBM DNA from normal EBV seropositive adults

Semi-quantitative PCR was performed on DNA from single PBM samples from 36 healthy adults. Figures show autoradiographs obtained following PCR on 10 representative samples.

Figure 4-13a. Bam W PCR

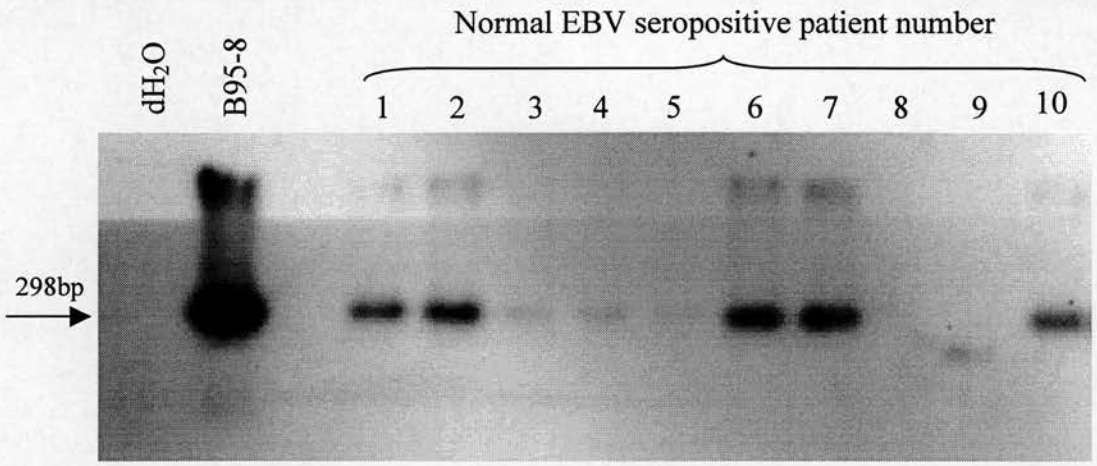


Figure 4-13b. β-globin PCR

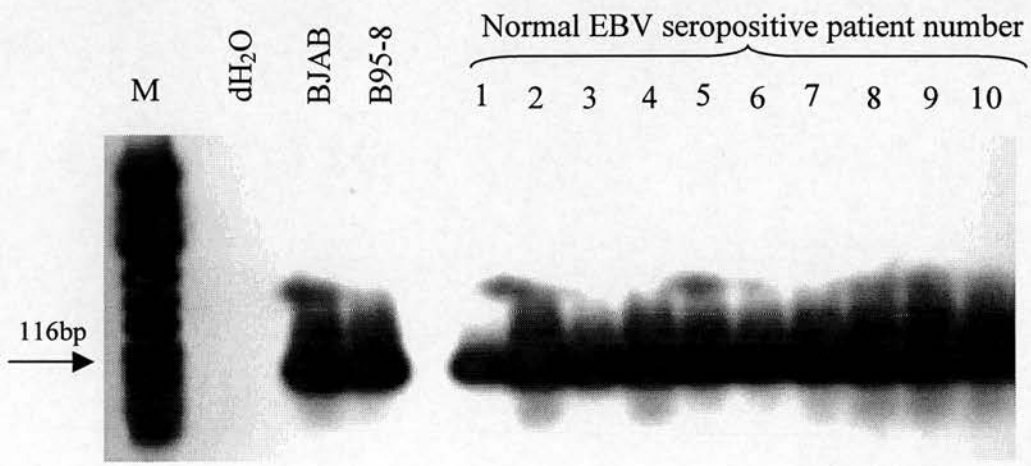


Table 4-1. EBV copy number in normal healthy adult EBV seropositives

Sample number	EBV genome copy number (genomes/10^6 PBMs)
1	50
2	<1
3	<1
4	50
5	1
6	75
7	1
8	1
9	5
10	200
11	100
12	150
13	125
14	<1
15	<1
16	10
17	100
18	10

Sample number	EBV genome copy number (genomes/10^6 PBMs)
19	5
20	<1
21	<1
22	<1
23	<1
24	10
25	<1
26	<1
27	10
28	<1
29	10
30	10
31	<1
32	<1
33	<1
34	<1
35	<1
36	50

Median EBV copy number =1 genome/ 10^6 PBMs.

Interquartile range=1-40 EBV genomes/10^6 PBMs

Table 4-2. EBV genome copy number in EBV seronegative adults

EBV Seronegative Adults	EBV copy number (Genomes/ 10^6 PBMs)
n=3	Undetectable (<1)

4.1.2.2 Multiple samples from normal healthy individuals

To assess whether EBV load in an individual fluctuates over time, EBV quantitation was carried out on multiple samples taken over a two year period from five normal healthy EBV seropositive adults. Autoradiographs of β-globin and Bam W PCRs from serial samples from three patients are shown in Figures 4-14a and b respectively, and the results summarised in Table 4-3.

The results show that EBV load remains consistently within the range detected in single samples from normal EBV seropositives of between <1 to 200 EBV genomes/ 10^6 PBMs (Table 4-1) over extended periods of time.

Figure 4-14a. β–Globin PCR on serial samples from normal EBV seropositives.

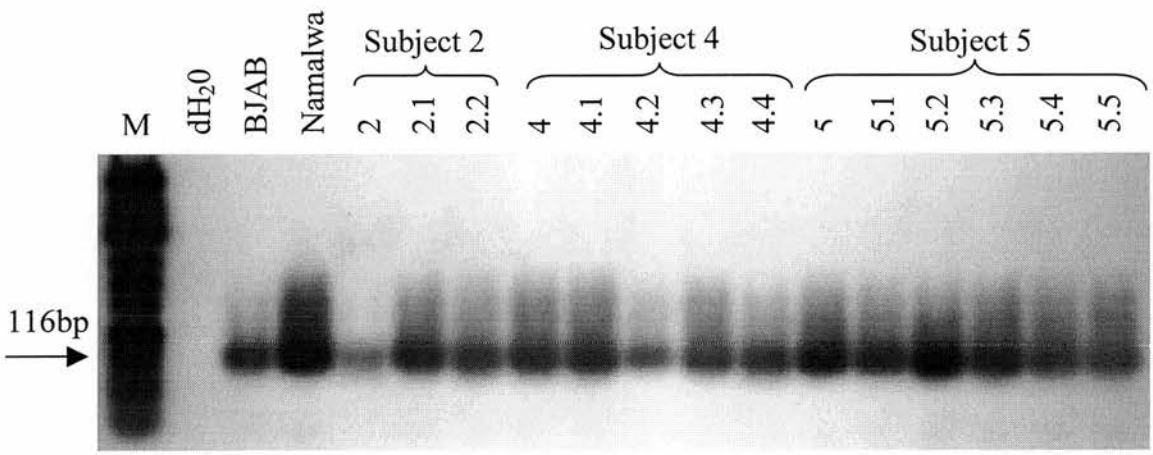
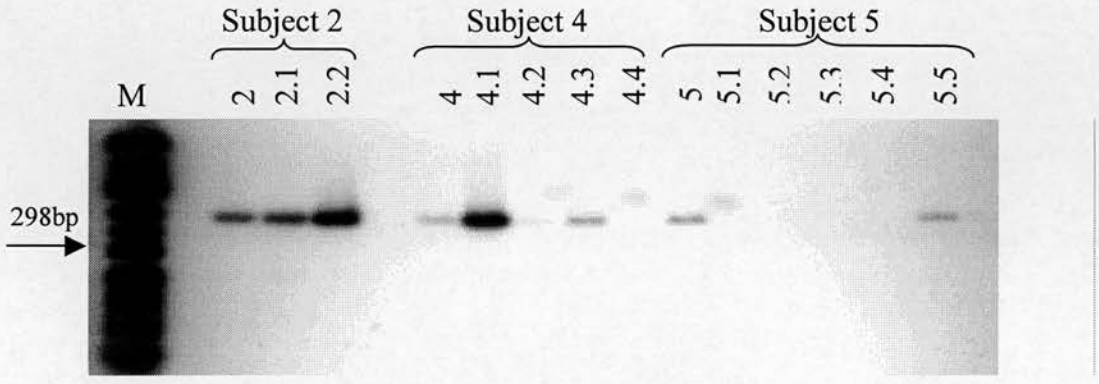


Figure 4-14b. Bam W PCR serial samples from normal EBV seropositives.



4.1.2.3 EBV copy number acute infectious mononucleosis (IM)

EBV semi-quantitative PCR was performed on DNA extracted from PBMs from 30 adults undergoing primary infection with EBV with acute IM aged between 17 and 34 years (mean 21.4 years). 40% of the patients tested were male, 60% female. Patients reported suffering from symptoms typical of IM including malaise and sore throat for periods ranging between two days and three months (mean 23 days) before visiting their general practitioner when blood samples were taken for serological diagnosis and to provide PBMs for this study. Patients were diagnosed as having acute IM if anti-VCA IgM antibodies were detected and the sample was monospot positive. Only single samples were available for testing from each patient. Representative autoradiographs of Bam W and β-Globin PCRs are shown in Figure 4-15a and b, and the results are presented in Table 4-4.

EBV DNA was detected in 27 of the 30 samples tested (90%) with a PCR sensitivity of 10 EBV genomes/10^6 PBMs. The EBV copy number detected in patients with acute IM ranged between undetectable (<10 genomes/10^6 PBMs) to 1 x 10^6 genomes/10^6 PBM. Median EBV load in acute IM was 1 x 10^4 genomes/10^6 PBMs (mean= 5.8 x 10^4 genomes/10^6 PBMs. Interquartile range= 40-1.5 x 10^4 genomes/10^6 PBMs). EBV load in patients with IM is significantly higher than that

in normal EBV seropositive adults (p=0.001 by Mann Whitney U test). There was no correlation between EBV load and duration of symptoms of IM as reported to GPs (p=0.7 by Spearman's correlation), indicating that early acute disease is not associated with higher EBV load and patients with extended illness did not show signs of reduced EBV load.

Table 4-3. Temporal changes in EBV copy number in normal adults

Subject	Time of sample (days after first)	EBV copy number *	Mean EBV copy number*
1	0	<1	<1
1.1	190	<1	
1.2	323	<1	
1.3	882	<1	
1.4	932	<1	
2	0	<1	10
2.1	455	5	
2.2	492	5	
2.3	770	30	
3	0	<1	<1
3.1	102	<1	
3.2	290	<1	
3.3	382	<1	
4	0	10	15
4.1	87	50	
4.2	429	1	
4.3	1155	5	
4.4	1211	10	
5	0	10	23
5.1	88	10	
5.2	182	10	
5.3	425	50	
5.4	522	5	
5.5	1253	50	

* EBV copy number in genomes/ 10^6 PBMs. n/d: not detected

Figure 4-15a. β–Globin PCR on PBM DNA from patients with acute infectious mononucleosis.

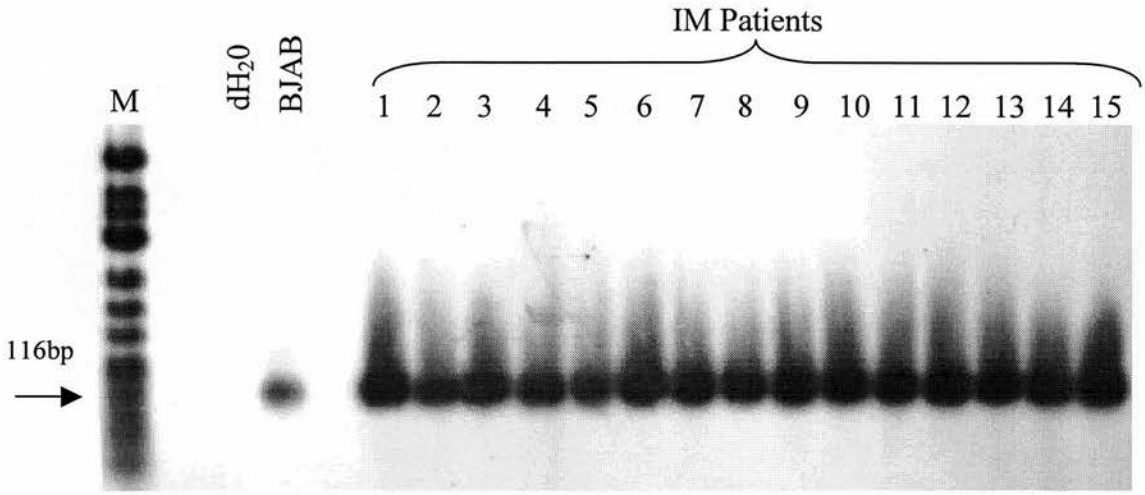


Figure 4-15b. Bam W PCR on PBM DNA from patients with acute infectious mononucleosis

Table 4-4. Clinical details and EBV genome copy number in PBMs from patients with infectious mononucleosis.

Patient number	Age (Years)	Sex	Duration of symptoms prior to sample (Days)	EBV copy number (genomes/ 10^6 PBMs)
1	20	M	4	4×10^5
2	21	M	n/a	3×10^5
3	24	M	4	550
4	34	F	21	200
5	17	F	2	650
6	20	M	4	200
7	22	F	2	350
8	29	M	14	<10
9	23	M	7	1×10^6
10	18	F	14	600
11	20	F	18	30
12	21	F	18	40
13	21	F	2	200
14	19	F	90	2.75×10^3
15	22	F	14	60
16	19	F	90	<10
17	19	M	14	<10
18	25	F	3	60
19	23	M	60	1
20	20	F	39	200
21	21	F	30	1
22	19	F	90	1
23	19	F	14	200
24	22	F	14	1.75×10^3
25	26	M	21	600
26	19	F	30	10
27	22	M	7	1.7×10^4
28	19	M	60	6×10^3
29	21	M	14	1.5×10^3
30	21	F	4	1×10^3
Total n=30	17 to 34 Mean= 21.4	40% M 60% F	2 days to 3 months Mean= 23 days	Range: <10 to 1×10^6 Median= 1×10^4

4.1.2.4 EBV genome copy number in PBMs from patients with PTLD

EBV genome copy number was determined in PBM samples from 15 transplant recipients with EBV positive PTLD. Results and patient details are shown in Table 4-5 and a representative Bam W PCR autoradiograph shown in Figure 4-16.

Twelve cases of PTLD occurred following kidney transplant, two in liver transplants and one heart transplant. The samples were taken at the time of PTLD diagnosis, occurring between 60 days and 16 years post-transplant (Mean=4.76 years). EBV copy number detected in PBMs from patients with PTLD ranged from 400 to 1 x 10^6 EBV genomes/10^6 PBMs (median=1 x 10^4 genomes/10^6 PBMs. Interquartile range=3500-1 x 10^6 genomes/10^6 PBMs).

Figure 4-16a. Bam W PCR on PBM DNA from patients with PTLD

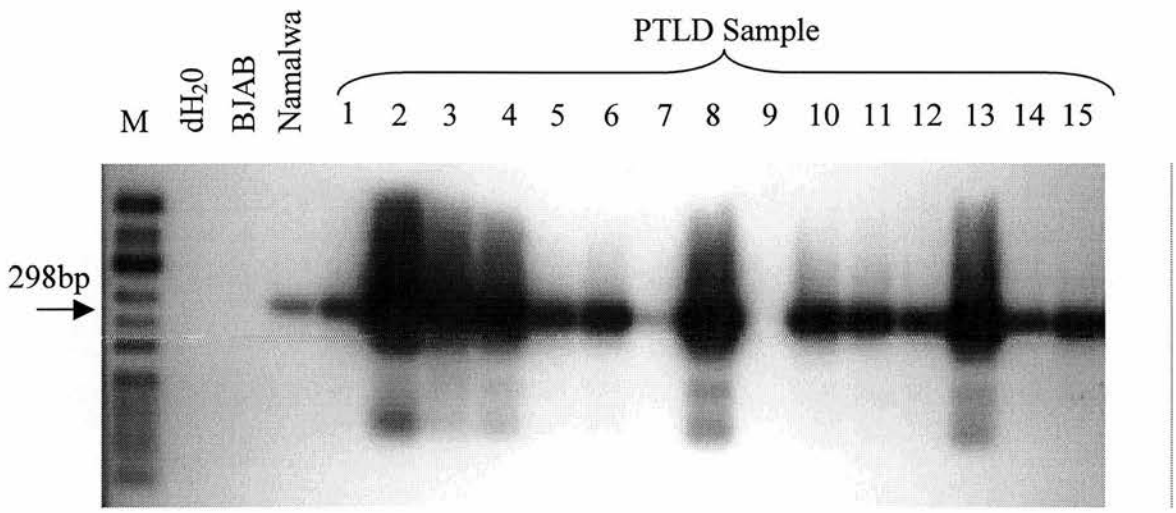


Figure 4-16b. β-globin PCR on PBMs from patients with PTLD

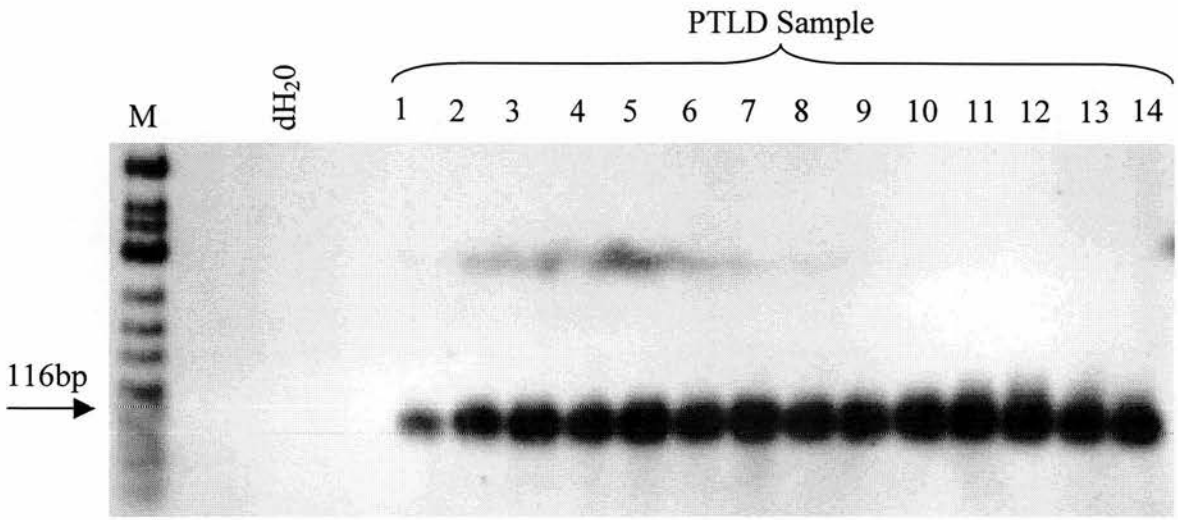


Table 4-5. Clinical details and EBV genome copy number in PBMs from patients with PTLD.

Patient Number	Transplanted organ	Histological diagnosis*	Time post transplant (days)	EBV load (Genomes/ 10^6 PBMs)
1	Kidney	MM	1924	27 000
2	Kidney	IB	1297	60 000
3	Kidney	PH	539	7 000
4	Kidney	n/a	2245	2 250
5	Kidney	IB	1989	30 000
6	Kidney	n/a	2950	10 000
7	Kidney	G-LN	74	400
8	Kidney	IB	5879	100 000
9	Kidney	IB	3137	600
10	Kidney	IB	246	5 000
11	Kidney	IB	267	1 000 000
12	Kidney	IB	754	15 000
13	Heart	IB	217	50 000
14	Liver	IB	60	3 300
15	Liver	IB	496	3 800
Totals			**Mean Time to PTLD**	**Mean EBV Copy No.**
Kidney	12		1775	104 770
Heart	1		217	50 000
Liver	2		278	3 550
Totals	**15**		**Mean= 1738 days (4.76 years)**	**Median= 1 x 10^4 genomes/ 10^6 PBMs Interquartile range=3500 – 1 x 10^6genomes/10^6 PBMs**

PTLD diagnosis key: MM= multiple myeloma. IB=immunoblastic lymphoma.
PH= plasmacytic hyperplasia. G-LN=granulomatous lymphadenopathy.
n/a= not available.

4.1.2.5 EBV genome copy number in PBMs from cardiothoracic transplant recipients

This study was designed to investigate the progression of EBV infection in cardiothoracic transplant recipients. Between January 1995 and November 1999 132 patients preparing to undergo heart, lung or combined heart and lung transplantation were recruited for this study from Harefield Hospital, Middlesex. A heparinised blood sample was taken, providing a 'pre-transplant' sample for analysis. Following transplantation, blood samples were taken at intervals on outpatient visits and patients were followed up where possible for the entire duration of the study. Patients were lost to follow up through death, or through no longer attending outpatient clinics at Harefield Hospital. Up to thirteen samples were obtained from each patient (mean of 5 samples) with a follow up time of up to 1110 days from the date of transplant (mean 415 days).

4.1.2.5.1 Pre-transplant samples

EBV genome copy number was assessed in PBMs isolated from 132 patients immediately prior to surgery for heart, lung or combined heart and lung transplantation (aged 20.3 to 71.2 years, median 47.2 years, 64% male, 36% female). Twelve of these patients had their transplants cancelled and were therefore not followed up, but their 'pre-transplant' EBV copy number data is included. Patient details and pre-transplant EBV copy number is summarised in Table 4-6.

EBV DNA was detected in pre-transplant samples from 85 of 132 patients tested (64.4%) with EBV genome copy number ranging from 1 to 200 EBV genomes/ 10^6 PBMs. EBV DNA was not detected in the remaining 47 patients (35.6%) indicating an EBV copy number of <1 genome/ 10^6 PBMs. The overall median EBV load was 5 EBV genomes/10^6 PBMs. There was no significant difference in pre-transplant EBV load according to transplant type.

Three transplant recipients were EBV seronegative pre-transplant, but all seroconverted by the time their first post-transplant sample was obtained. The source of infecting virus was not determined.

Table 4-6. Pre-transplant EBV copy number and clinical details of patients awaiting transplantation.

Transplant type	Number of patients	Age Range (Years)	Sex (%) M	F	EBV copy number (genomes/ 10^6 PBMs)
Lung	34	19.44 - 60.50 Median= 51.24	62	38	0 - 60 Median = 6.5
Heart	58	20.67 - 71.21 Median= 50.98	59	41	0 - 200 Median = 2
Heart and Lung	28	17.34 - 47.72 Median= 32.36	79	21	0 - 70 Median = 5
Transplant cancelled	12	20.3 - 56.79 Median= 48.66	65	35	0 - 30 Median = 5
Totals	**132**	**17.34 - 71.2 Median= 47.10 years**	**64**	**36**	**0 - 2 00 Median=5**

4.1.2.5.2 EBV genome copy number following transplantation

EBV DNA quantitation was performed on samples taken at intervals following organ transplantation from 96 patients. Patients were followed for up to 1110 days after transplantation with between one and thirteen post-transplant samples obtained from individual patients (median of 5 samples per patient). DNA extracted from PBMs was subjected to EBV semi-quantitative PCR analysis. This data is summarised in Table 4-7. Southern blots showing EBV detection in serial samples from two representative patients (an EBV seropositive and an EBV seronegative who seroconverted following transplantation) are shown in Figures 4-17 to 4-20.

EBV DNA was detected in at least one post-transplant sample from 84 of the 96 patients tested (87.5%). Post-transplant EBV copy number ranged from

undetectable (<1 genome/10^6 PBMs) to 1 x 10^5 genomes/10^6 PBMs (mean=1092 genomes/10^6 PBMs, median=10 genomes/10^6 PBMs). In individual patients, the highest EBV copy number was detected between 1 day and 755 days post-transplant. EBV load exceeded the range detected in normal EBV positive donors in at least one post-transplant sample from 44 transplant recipients (46%). In 12 patients (13%) EBV load reached levels equivalent to PTLD (3500- 1 x 10^6 genomes/10^6 PBMs) and in 32 (33%) patients EBV load reached levels equivalent to patients with IM (40-1.5 x 10^4 genomes/10^6 PBMs). EBV load remained in the range detected in normal individuals (<1-40 EBV genomes/10^6 PBMs) in 52 transplant recipients (54%).

The 12 patients with EBV loads equivalent to that in PTLD were 8 males, 4 females aged 17-60 years (mean 43 years). Two received lung transplants, 6 heart transplants and 4 combined heart/lung. The highest EBV load (1 x 10^5 genomes/10^6 PBMs) was detected in a 17 year-old EBV seronegative patient given combined heart/lung transplant for cystic fibrosis. Her first post-transplant sample was taken 9 months after surgery, by which time she had seroconverted for EBV, with an EBV copy number of 50 genomes/10^6 PBMs. The source of EBV infection is not known, although EBV DNA was not detected in archival spleen tissue from the 11-year-old organ donor. The patient's EBV copy number fluctuated (Figure 4-20) but showed a general increase to reach a maximal level of 1 x 10^5 genomes/10^6 PBMs by 1 year post-transplant, with the same level of EBV load detected in 2 of 3 further samples taken up to 18 months post-transplant when the patient died from chronic graft rejection and organ failure. EBV load increased to this maximum level following switching of the principal immunosuppression from cyclosporin to FK506. There was no evidence of PTLD in this patient despite detection of EBV load within the range in patients with PTLD. Autoradiographs of Bam W and β-globin PCRs and a graph of changes in post-transplant EBV load and drug dose are shown in Figures 4-19 and 4-20.

Underlying factors were investigated using Spearman's correlation to determine associations with whether EBV loads in transplant recipients reached levels equivalent to those seen in IM or PTLD. There was no association with age ($p=0.327$), sex ($p=0.532$) or transplanted organ ($p=0.821$).

Table 4-7. Post-transplant EBV genome copy number and patient details.

Transplant type	Number of patients	Age Range (Years)	Sex (%)		EBV copy number. (genomes/ 10^6 PBMs)
			M	F	
Lung	27	19.44 - 60.50 Median= 51.94	65	35	<1 to 5 x 10^3 Median= 10
Heart	46	20.67 - 65.36 Median= 50.60	60	40	<1 to 1 x 10^5 Median= 10
Heart and lung	23	17.34 - 47.72 Median= 33.79	80	20	<1 to 1 x 10^5 Median = 17.5
Total	**96**	**17.34 - 65.36** **Median= 46.80**	**66**	**34**	**0 to 1 x 10^5** **Median= 12**

Figure 4-17a & b. Bam W and β-Globin PCR on serial samples from an EBV seropositive cardiothoracic transplant recipient.

Samples shown in these blots include the Pre-transplant sample (Pre-Tx) and subsequent samples obtained following transplantation (labelled .1 to .12). A graph of EBV load in these samples and its change over time is shown in Figure 4-18.

Figure 4-17a. Bam W PCR

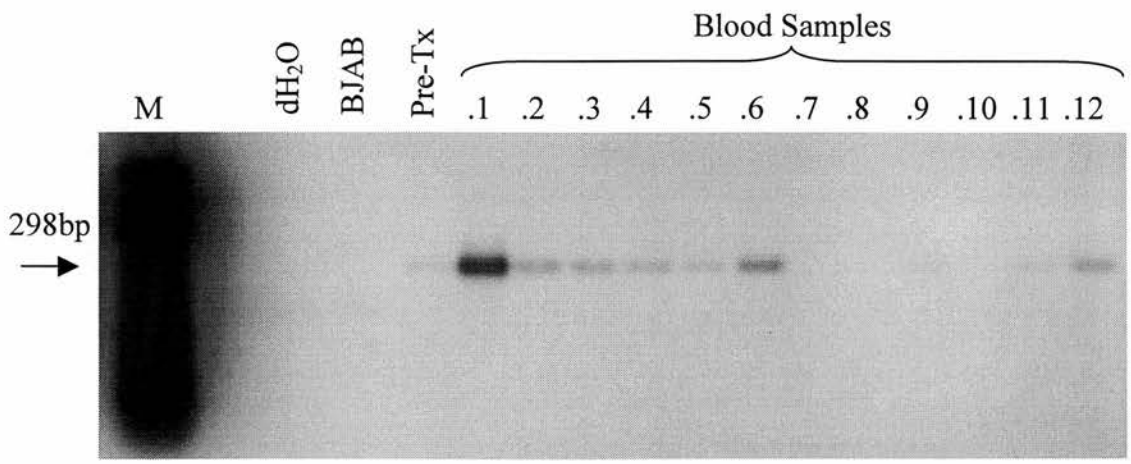


Figure 4-17b. β-Globin PCR

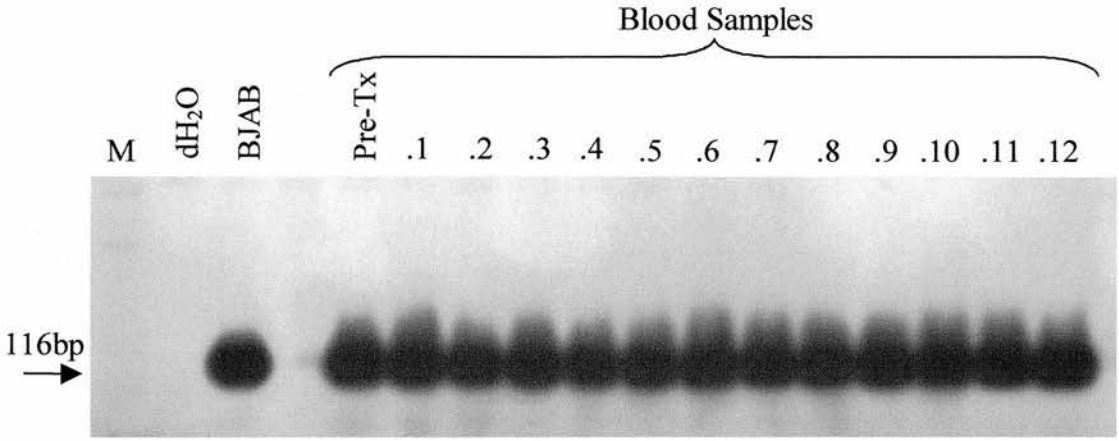
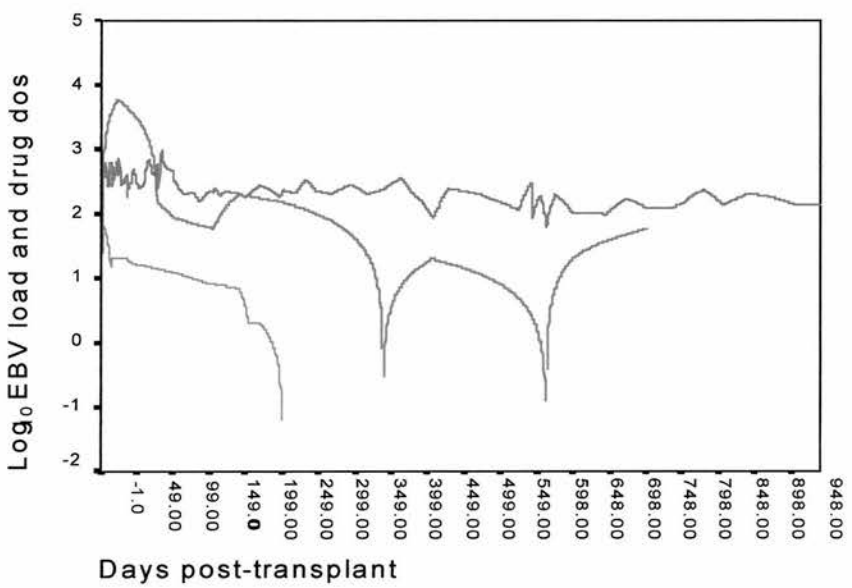


Figure 4-18. Graph of EBV load and immunosuppressive drug dose in an EBV seropositive transplant recipient.

EBV load (log_{10} of EBV genome copy number/ 10^6 PBMs) and levels of cyclosporin (CsA) and prednisolone plotted against time in serial samples from an EBV seropositive transplant recipient (EBV load data derived from autoradiographs presented in Figure 4-17a & b).



Key:

EBV genome copy number

Cyclosporin dose

Prednisolone dose

Figure 4-19. Bam H1 W and β-Globin PCR on serial samples from an EBV seronegative cardiothoracic transplant recipient.

Autoradiographs of Bam H1 W and β-Globin PCR on serial samples from a patient who was EBV seronegative pre-transplant, and seroconverted following transplantation. Samples included were taken pre-transplant (Pre-Tx) and at intervals post transplant (samples .1 to .10). A graph of EBV load and drug dose in these samples is shown in Figure 4-20.

Figure 4-19a. Bam H1 W PCR.

A positive control was included in the PCR reaction, but was not run on the gel from which this blot was derived.

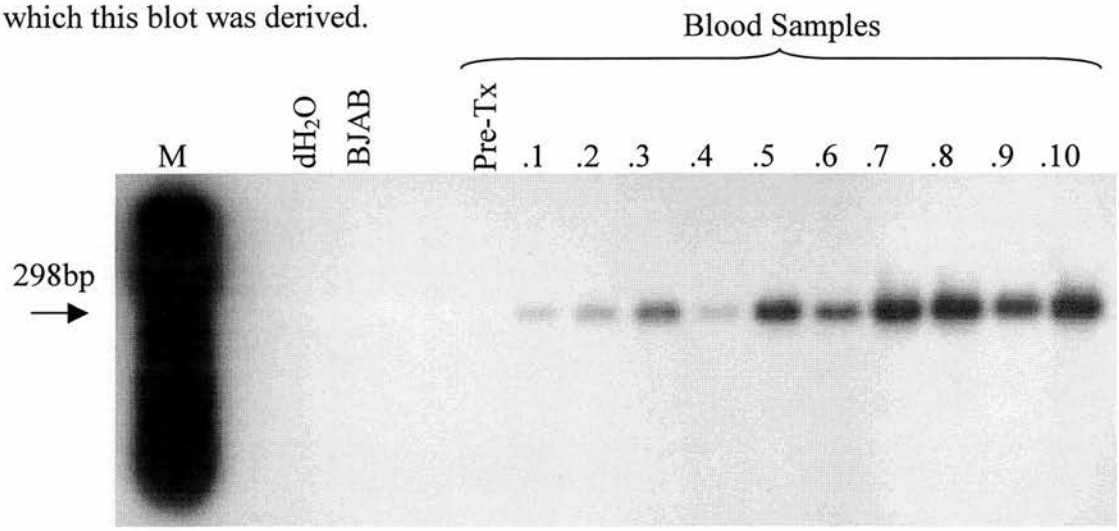


Figure 4-19b. β-globin PCR.

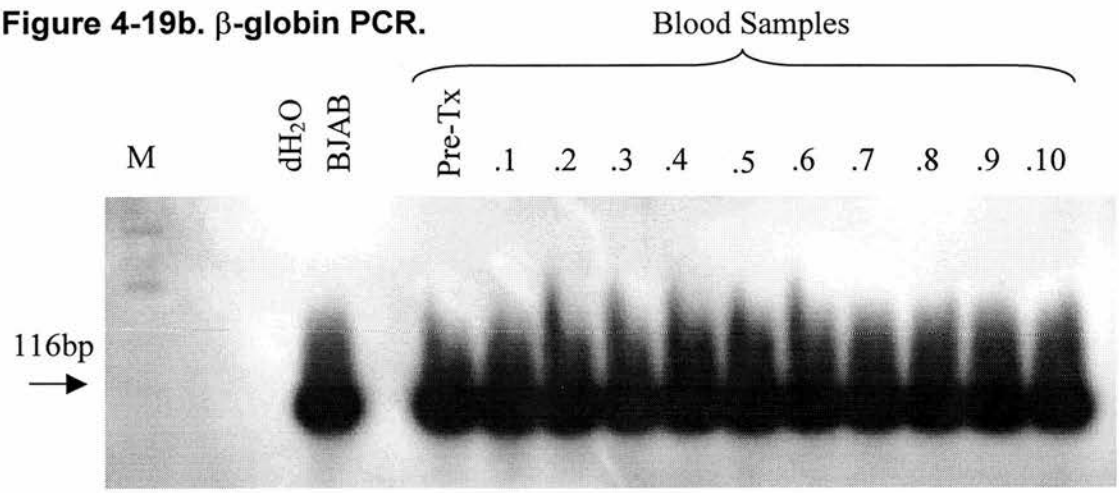
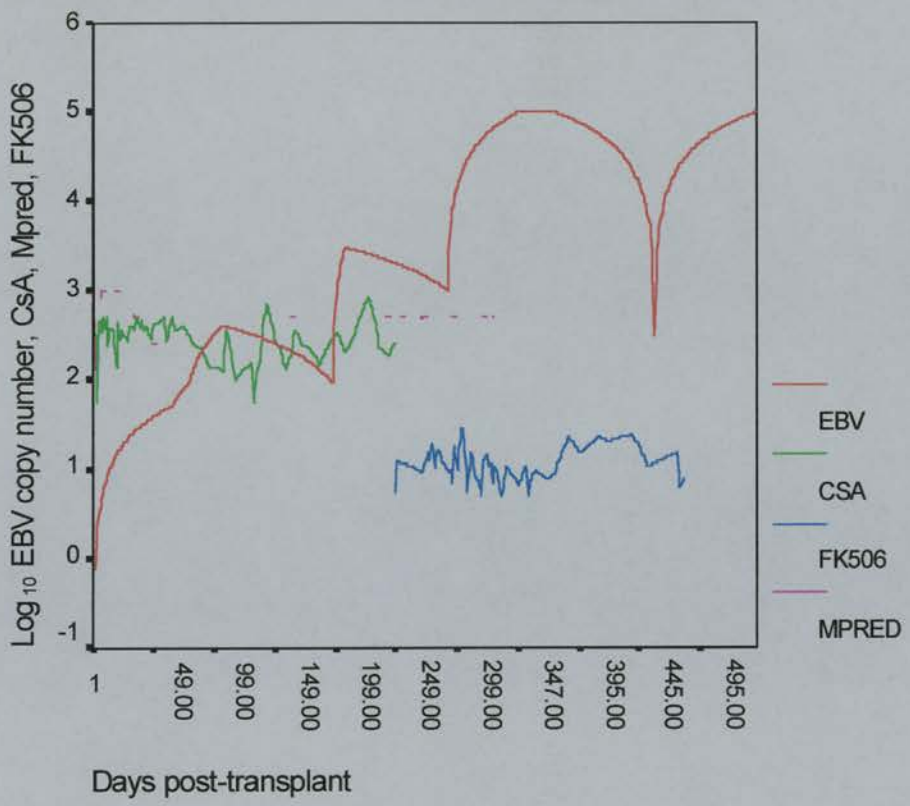


Figure 4-20. Graph of EBV load and immunosuppressive drug dose in an EBV seronegative transplant recipient.

EBV load (log_{10} of EBV genome copy number/ 10^6 PBMs) and levels of cyclosporin (CsA), Methylprednisolone (Mpred) and FK506 plotted against time in serial samples from an EBV seronegative transplant recipient (EBV load data derived from autoradiographs presented in Figure 4-19a & b).



4.1.3 Statistical analysis of EBV quantitation data

The results of EBV quantitation in control groups (normal EBV seropositives and patients with IM and PTLD) and patients undergoing transplantation were analysed to identify statistical differences between these groups. Results of these analyses are presented in this section.

4.1.3.1 EBV load pre-transplant in patients awaiting cardiothoracic transplantation

EBV load in patients awaiting transplantation were compared on the basis of sex, age and organ to be transplanted, using the Mann Whitney U test to compare the median values of pairs of groups (Table 4-8). There was no significant difference between each transplant type in pre-transplant EBV load ($p>0.5$). There was no significant difference in the distribution of patient age, sex or EBV load in different transplant groups by the Kruskall-Wallace test, shown in Table 4-9. The Kruskall-Wallace test is a non-parametric test to determine whether several independent samples have the same distribution.

Table 4-8. Comparison of EBV load in pre-transplant samples using Mann Whitney U test

Comparisons of groups	EBV load pre-transplant
Lung : Heart	$p = 0.649$
Heart: Heart and lung	$p = 0.972$
Lung : Heart and lung	$p = 0.724$

EBV load was compared between patients with different transplanted organs using the Mann Whitney U test. EBV load in each group is shown in Table 4-6.

Table 4-9. Results of Kruskall-Wallace test on overall distribution of age, sex and pre-transplant EBV load with transplant types.

	Grouping variable		
	Transplant type	**Sex**	**Age**
Test group			
EBV load pre-transplant	p = 0.895	p = 0.465	p = 0.459

Patients receiving different organ transplant types were compared on the basis of pre-transplant EBV load, sex and age at transplant. There was no significant difference in these factors with different transplant types.

4.1.3.2 Comparison of EBV copy number in normal controls, patients with IM, patients with PTLD and pre- and post-transplant samples.

To assess the overall distribution of EBV load in transplant recipients and patients with EBV-associated diseases, Mann-Whitney U tests were used to compare the medians of EBV copy number between patient groups. Results are summarised in Table 4-10. EBV load in patients awaiting transplantation is equivalent to that in normal EBV seropositives (p=0.542), demonstrating that overall, EBV load in transplant recipients is within the normal range prior to surgery. EBV load in PBMs from patients with PTLD is significantly higher than that in patients with IM ($p<0.001$). However there is overlap in the range of EBV load detected in patients with IM and PTLD, demonstrating the difficulty in distinguishing between the two diseases on the basis of EBV load. EBV load in IM and PTLD is significantly higher than in normal EBV carriers and patients awaiting transplantation ($p< 0.001$ for all tests). Therefore EBV associated disease is associated with significantly increased EBV load. In post-transplant samples, EBV load in individual patients ranged from 5 to 1×10^6 EBV genomes/ 10^6 PBMs (median of EBV load in all post-transplant

samples = 10 EBV genomes/ 10^6 PBMs, mean= 1319 genomes/ 10^6 PBMs), and overall showed a highly significant difference from pre-transplant levels ($p<0.001$).

The number of transplant recipients with EBV loads in the interquartile range detected in EBV associated diseases is shown in Table 4-11. In 18 transplant recipients, the pre-transplant EBV load was within the interquartile range detected in patients with IM. However, the highest EBV load detected pre-transplant was 200 genomes/10^6 PBMs, a level detected in normal donors. Post-transplant, 12 patients (14%) had EBV loads within the range detected in patients with PTLD and levels equivalent to IM in 34 patients (40%). In 38 patients (45%) EBV loads remained in the range detected in normal healthy carriers.

Using linear regression analysis there was a significant association between EBV copy number and time post-transplant ($p=0.013$). A scatter plot of EBV load against time for all samples, both pre- and post-transplant, is shown in Figure 4-21 showing a positive correlation between time post-transplant and EBV load (Pearsons' correlation coefficient $r= 0.521$).

Table 4-10. Comparison of distribution of EBV copy number between patient groups.

Table shows the results of Mann-Whitney U tests to compare EBV load pre- and post-transplant with the levels detected in controls and patients with EBV associated disease. Pre- and post transplant EBV loads assessed were the combined values for all samples. EBV loads for each group are shown as range and median, expressed as EBV genomes/10^6 PBMs.

Column 1 Range (median)	**Column 2** Range (median)	**Mann-Whitney U Test** (comparison of columns 1 & 2)
Pre-transplant 0-200 (3)	Post-transplant 0-1 x 10^5 (10)	P<0.001
	Normals 0-200 (1)	P=0.542
	IM 0-1 x 10^6 (1 x 10^4)	P=0.001
	PTLD	P=0.001
Post-transplant 0- 10^5 (10)	Normals 0-200 (1)	P<0.001
	IM 0-1 x 10^6 (1 x 10^4)	P<0.001
	PTLD 400-1 x 10^6 (1 x 10^4)	P<0.001
IM 0-1 x 10^6 (1 x 10^4)	PTLD 400-1 x 10^6 (1 x 10^4)	P=0.001

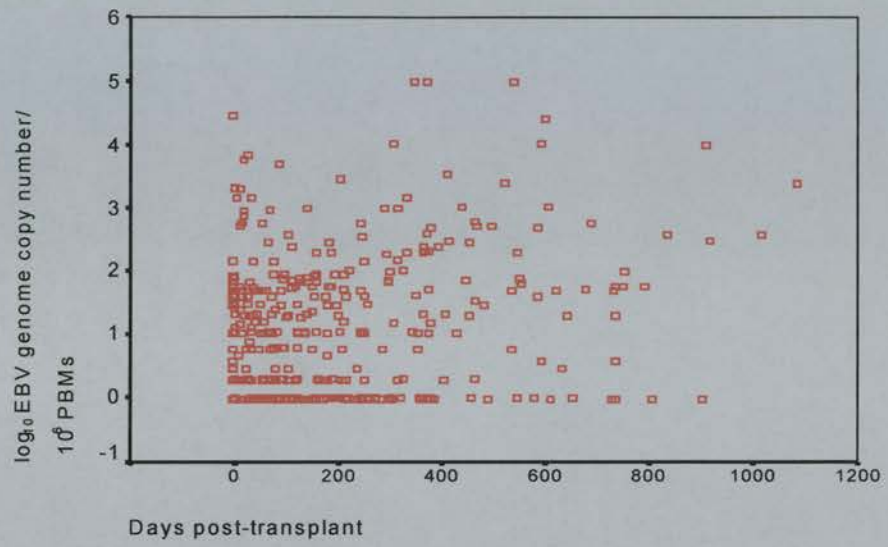
Table 4-11. Transplant recipients with EBV copy number equivalent to that in EBV associated conditions

Table shows the number and percentage of transplant recipients with at least one sample having an EBV load within the interquartile range determined for other patient and control groups.

	Range and interquartile range of EBV load in control groups. Number and % of transplant recipients with EBV load within the same interquartile range as controls		
	Normal EBV carriers	**IM**	**PTLD**
Transplant recipients (EBV load (genomes/ 10^6 PBMs))	Range: 0-200 Interquartile range: 0-40 genomes/ 10^6 PBMs	Range: 0 – 1 x 10^6 Interquartile range: 40-15000 genomes/10^6 PBMs	Range: 400–1 x 10^6 Interquartile range: 3500-45000 genomes/ 10^6 PBMs
Pre- transplant (Range: 0-200 EBV genomes/ 10^6 PBMs)	64 (78%)	18 (22%)	0
Post-transplant (0- 1 x 10^5 EBV genomes/ 10^6 PBMs)	38 (45.2%)	34 (40.5%)	12 (14.3%)

Figure 4-21. Scatter plot of EBV genome copy number against sample date for all post-transplant samples

Dots represent individual measurements of 479 samples from 136 patients.



4.1.3.3 Influence of immunosuppression on EBV copy number

The influence of iatrogenic immunosuppression on EBV load in transplant recipients was investigated by assessing associations between EBV load and dose of immunosuppressive drugs received by patients in the month prior to taking blood samples for quantitative EBV PCR. Also the cumulative dose of each drug was assessed, that is the total dose received prior to sampling. The agents studied were cyclosporin A, azathioprine, prednisolone, methylprednisolone and FK506. These results are presented in Table 4-12.

Significant associations were found between mean daily dose of cyclosporin and increased EBV load when analysed by linear regression ($p=0.001$). Therefore increased dose of cyclosporin is associated with, and in part may be responsible for, increased EBV copy number following transplantation. There were also significant correlations between increased cumulative dose of cyclosporin, azathioprine and prednisolone and increased EBV load ($p= 0.021$, 0.003 and 0.002 respectively by linear regression analysis). This indicates that increased duration of exposure to the

standard immunosuppression regime is associated with increased EBV load, and confirms the data shown in Figure 4-21, whereby EBV load shows an increase with time post-transplant.

No significant associations were found using analysis of variance (ANOVA) of EBV load when drug doses were categorized as high, moderate or low, based on the interquartile range of doses. The two patients with the highest post-transplant EBV loads were both treated with FK506, but there was no significant association between EBV load and FK506 dose.

Table 4-12. Association between immunosuppressive drug dose and EBV genome copy number

Drug doses categorized from the interquartile range. Cumulative drug doses and associated EBV loads are not shown. Significant associations using linear regression analysis of continuous variables of EBV and drug dose are asterisked.

	Mean daily dose in preceding month			ANOVA (analysis of variance)	Linear regression	
		EBV load (genomes/10^6 PBMs)				
Drug	**Dose**	**Range**	**Mean**	**P value**	**P value** (daily dose)	**P value** (cumulative dose)
Cyclosporin	Low	0-7000	160.9	0.622	P=0.001*	p=0.021*
	Moderate	0-10000	167.9			
	High	0-25000	547.8			
Azathioprine	Low	0-25000	482.45	0.264	P=0.172	P=0.003*
	Moderate	0-7000	157.12			
	High	0-2000	90.18			
Prednisolone	Low	0-2000	82.01	0.775	P=0.102	P=0.002*
	Moderate	0-100000	3690.54			
	High	0-10000	907.39			
Methyl-prednisolone	Low	0-70	21.12	1.0	P=0.827	P=0.283
	Moderate	0-2000	79.43			
	High	-				
FK506	Low	20-100000	20308	1.0	P=0.543	P=0.450
	Moderate	5-100000	6799			
	High	0-1000	333.33			

4.2 Analysis of EBV gene expression by RT-PCR

Having shown that EBV load is significantly elevated post-transplant further experiments were carried out to determine whether this was due to lytic replication of EBV or expansion of B-lymphoblasts. This was done to establish whether the pattern of EBV gene expression in PBMs following organ transplantation could predict PTLD development.

cDNA derived from PBMs and tumour biopsies was analysed by PCR to amplify transcripts from the human β-actin gene and the EBV genes EBER1, LMP1, LMP2a/b, gp350 and EBNA3C. These PCR reactions were selected to provide an overall assessment of the pattern of EBV gene expression in a given sample, that is, detection of EBV gene expression (EBER1); latent membrane protein expression (LMP1, LMP2a and b); lytic replication (gp350), and full LCL-like latent antigen expression (EBNA 3c). As described in the introduction, three basic patterns of EBV gene expression were described by Rowe *et al* (1992), termed Latency I, Latency II and Latency III which are associated with distinct cell phenotypes in EBV infected cell lines and particular EBV associated diseases *in vivo*. EBERs transcripts are detected in all forms of EBV latency described. To summarise, Latency I is characterized by EBNA1 expression in the absence of other EBV latent genes and lytic replication and is seen in Burkitt's lymphoma tumour cells *in vivo* and at early passage *in vitro*. Latency II involves expression of EBNA1 in the absence of the other EBNAs, expression of LMP1 and sometimes LMP 2a and 2b but no lytic replication. Latency II is seen in nasopharyngeal carcinoma tumour cells. Latency III is characterized by expression of all latent antigens; that is EBNAs 1, 2, 3a, 3b and 3c, EBNA-LP, LMP1, 2a and 2b. This pattern is seen in immunoblastic PTLD tumour cells and in LCLs *in vitro*. In this study the terms Latency I, II and III are used to compare patterns of EBV gene expression to forms of EBV latency previously described. Detection of EBNA3c mRNA is taken as indicative of Latency III, since the EBNA transcripts are transcribed together from a single promoter. Detection of gp350 transcripts is taken to indicate EBV replication since this late lytic transcript is only expressed in cell undergoing productive infection.

4.2.1 Optimisation of RT-PCR conditions

The reaction conditions of each PCR were standardised to ensure optimal sensitivity and specificity of detection. Primer and probe sequences were derived from previously published sources or were designed for this study from the B95-8 prototype EBV sequence. Sequences are shown in Materials and Methods Table 3-2. Reaction conditions were optimised for $MgCl_2$ concentration and annealing temperature (Materials and Methods 3.8.1.6), and in the case of β-actin, for cycle number to provide a semi-quantitative assessment of the amount of amplifiable RNA present in a sample. Optimised reaction conditions are summarised in Table 4-13.

The sensitivity of each PCR was demonstrated through amplification of cDNA generated from RNA from a B95-8 dilution series. Specificity of each PCR was confirmed by PCR of cDNA generated from total cellular RNA extracted from EBV infected control cell lines and tumour material which exhibit a known characteristic pattern of EBV gene expression: c19, M148 and IB4. Representative autoradiographs of RT-PCRs showing the sensitivity and specificity of each reaction are shown in Figure 4-22. Nested RT-PCRs to amplify mRNA encoding EBERs, LMP1, LMP2a, LMP2b and EBNA3c detect transcripts in as few as 100 B95-8 cells in a background of 10^6 EBV negative cells. RT-PCR to detect the EBV lytic transcript gp350 detects transcripts in 10 B59-8 cells in 10^6 EBV negative cells.

Table 4-13 Summary of optimal $MgCl_2$ concentration and annealing temperature for RT-PCR amplification of EBV transcripts and human β-actin mRNA.

Gene	$MgCl_2$ concentration (mM)	Annealing temperature (°C)
β-Actin	1.5mM	64°C
EBERs	1mM	55°C
EBNA 3c 1st Round	1.5mM	50°C
EBNA 3c Nested	1.5mM	56°C
LMP1 1st Round	1mM	55 °C
LMP1 Nested	1.5mM	64 °C
LMP2a 1st Round	1.5mM	51 °C
LMP2a Nested	1mM	58 °C
LMP2b 1st Round	2mM	52 °C
LMP2b Nested	1mM	58 °C
gp350	3mM	53 °C

Figure 4-22 Southern blots demonstrating sensitivity and specificity of RT-PCR reactions

PCRs were performed on RNA from a dilution series of B95-8 with appropriate positive and negative control cells. The following controls were included in each PCR although individual blots for each PCR may not show all controls.

Lane annotation:

dH_2O: water control, dH_2O N: nested PCR water control (taken from 1st round product)

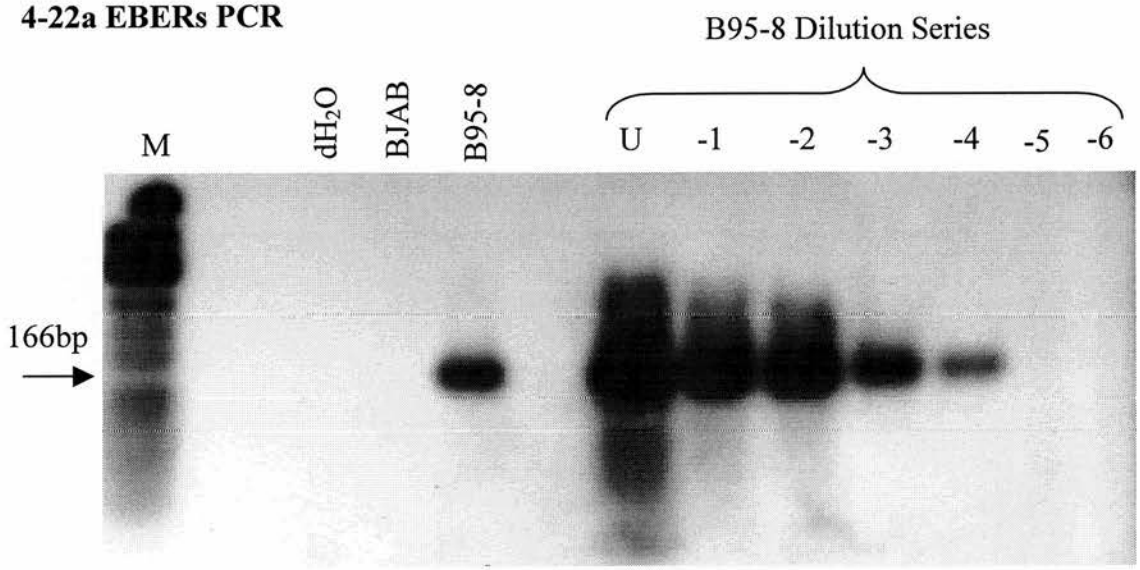
BJAB: EBV negative BL cell line. M148: EBV positive BL cell line:

C19: EBV positive NPC cell line. IB4: EBV positive BL cell line

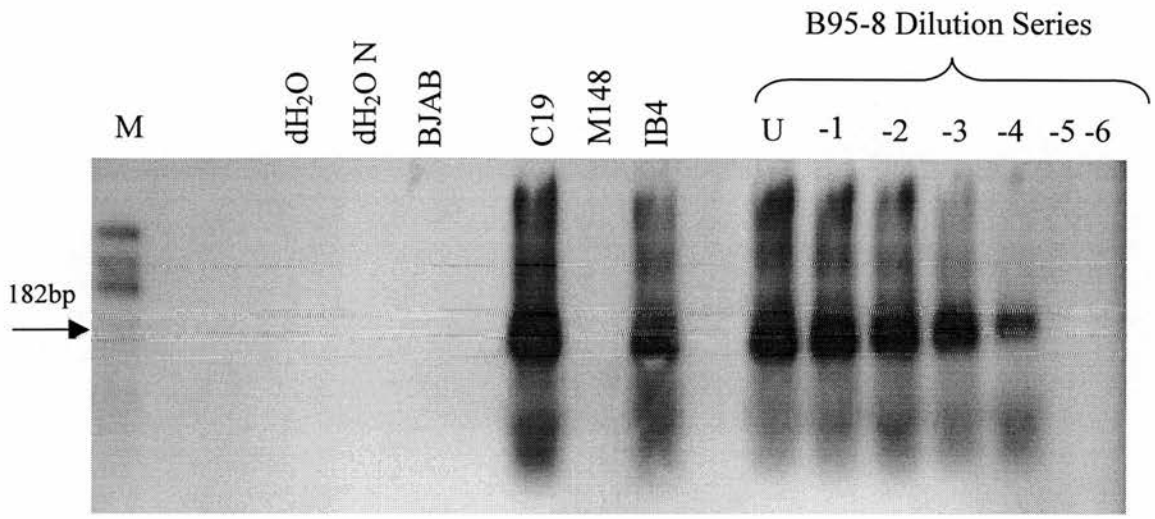
B95-8: EBV positive LCL

Dilutions: U: undiluted. –1: 10^5 B95-8/ 10^6 cells. –2: 10^4/ 10^6. –3: 10^3/ 10^6. -4: 10^2/ 10^6. –5: 10/ 10^6. –6: 1/ 10^6.

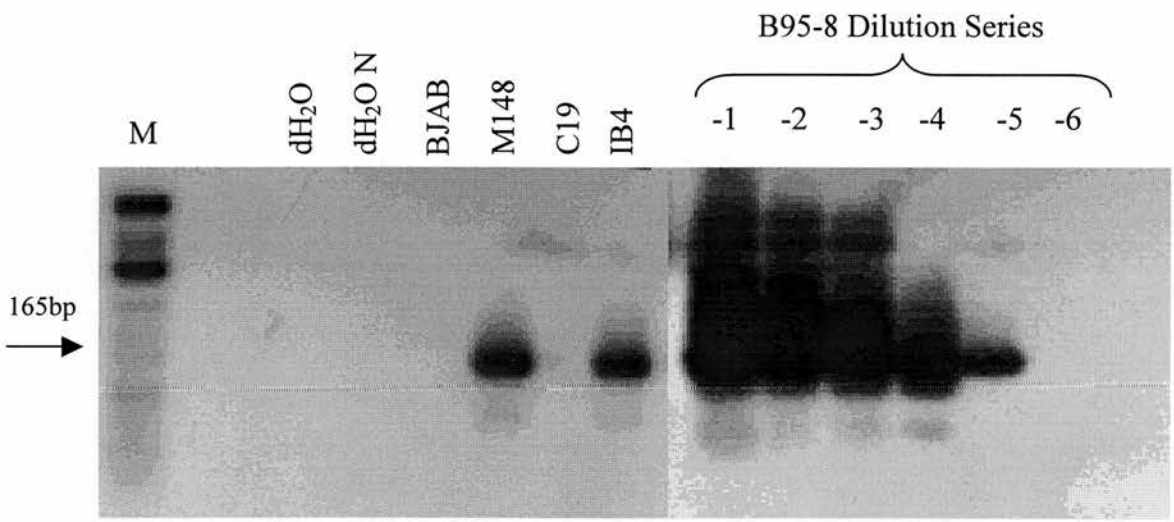
4-22a EBERs PCR



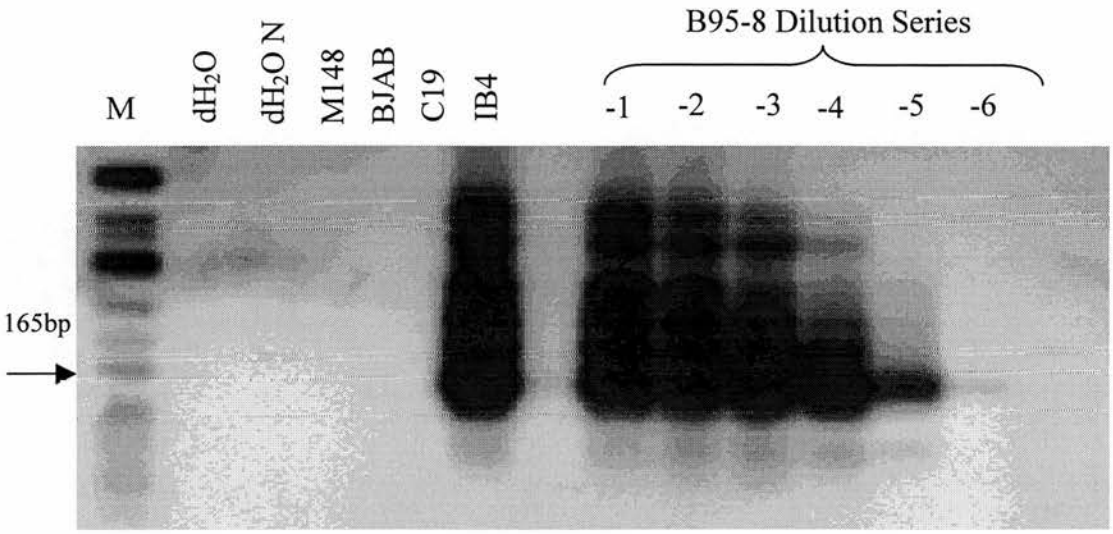
4-22b LMP1 Nested PCR



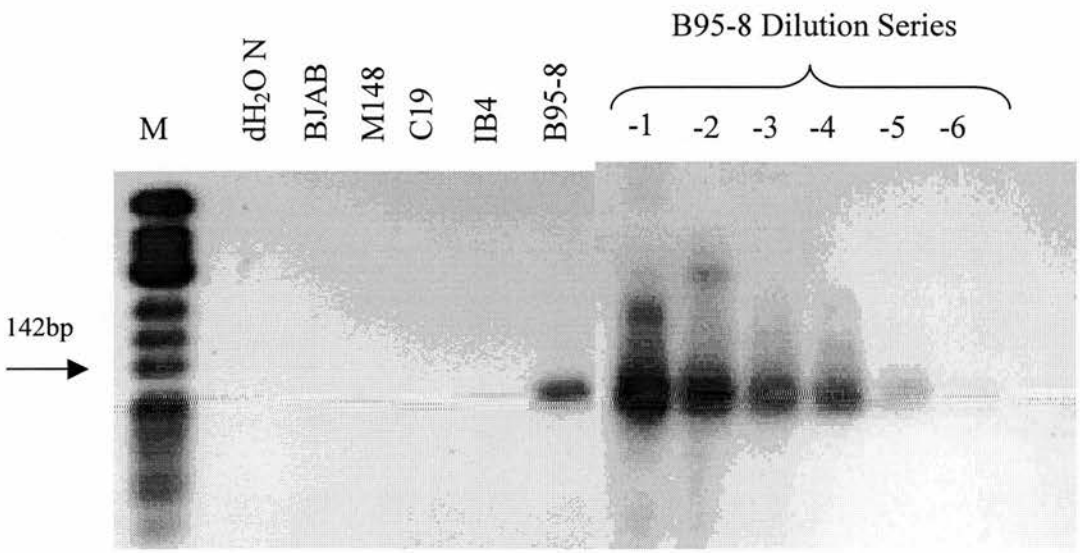
4-22c. LMP2a Nested PCR



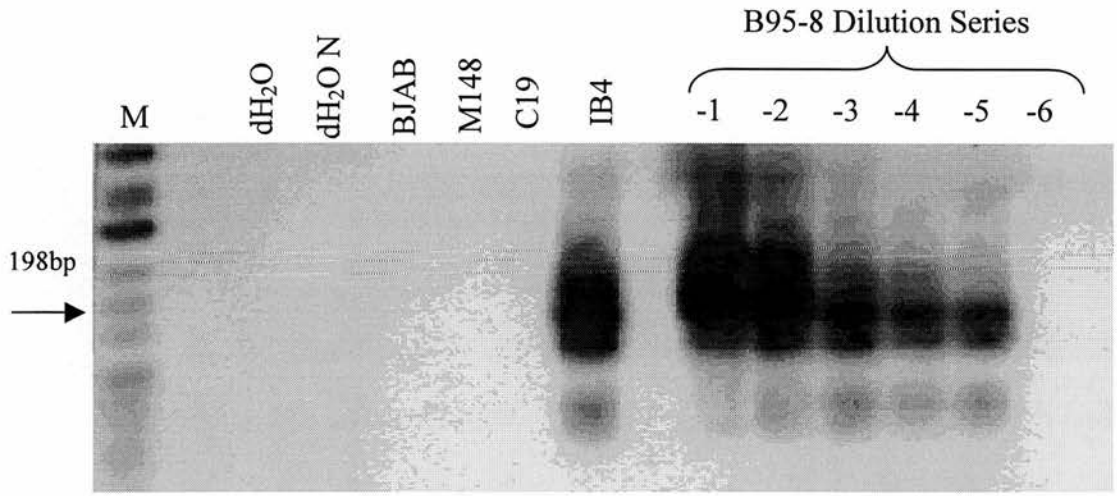
4-22d. LMP2b Nested PCR



4-22e. gp350 PCR



4-22f. EBNA 3c Nested PCR



4.2.2 EBV gene expression in normal healthy donors

RNA was extracted from PBMs isolated from heparinised blood from 36 normal healthy EBV seropositive adults and 3 EBV seronegatives. cDNA was generated as described in Materials and Methods section 3.3.1.4 from 1μg RNA and β-actin PCR performed on all samples to confirm that amplifiable cDNA was present (Figure 4-23a). RT-PCR for detection of EBV encoded transcripts were performed on β-actin positive RNA from all 36 donors to determine the pattern of EBV gene expression in PBMs from these individuals. Autoradiographs showing representative PCR analysis of each transcript are shown in Figures 4-23a to g and data for all normal donors is summarized in Table 4-14.

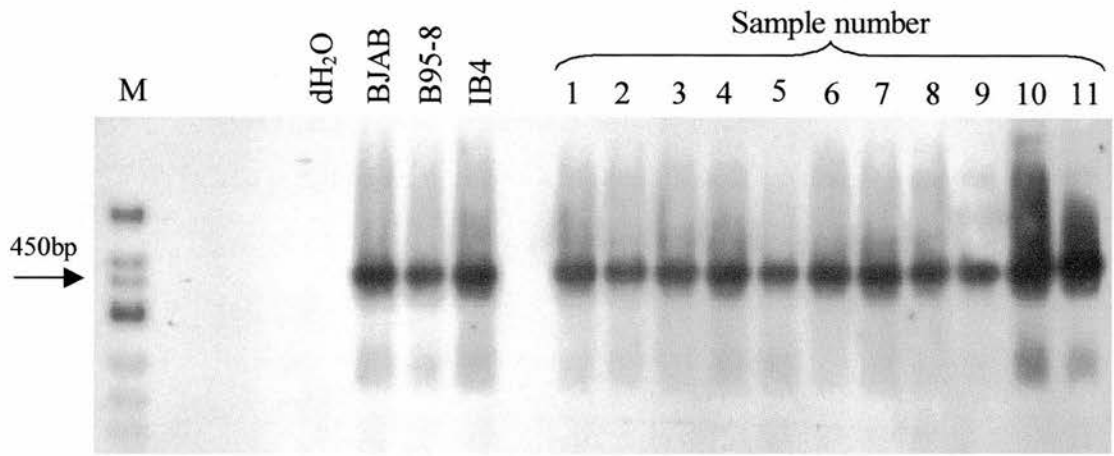
β-Actin mRNA was detected in the absence of any detectable EBV transcripts in 7 normal EBV seropositives. EBERs transcripts were detected in 28 of 36 samples (77.8%). EBERs encoding transcripts were the only EBV transcripts detected in 6 subjects. LMP2a-encoding transcripts were detected with EBERs in 20 subjects and LMP2a and b were detected in the same sample from 3 subjects. LMP1, EBNA3c or gp350 mRNAs were not detected.

Table 4-14. Summary of EBV gene expression in normal EBV seropositive and seronegative individuals

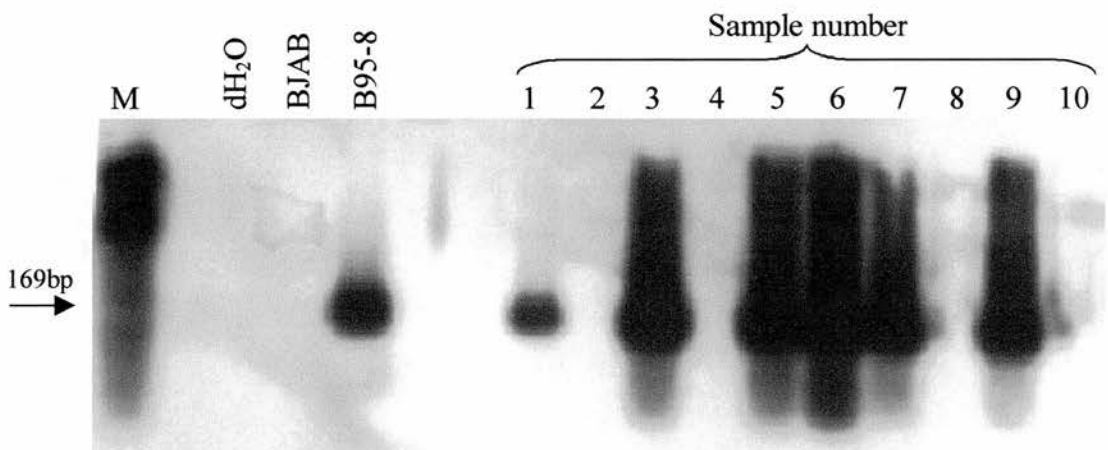
Sample no.	Actin	EBER1	LMP1	LMP2a	LMP2b	gp350	EBNA3c
1	+	-	-	-	-	-	-
2	+	+	-	-	-	-	-
3	+	+	-	+	-	-	-
4	+	+	-	+	+	-	-
5	+	+	-	-	-	-	-
6	+	+	-	-	-	-	-
7	+	+	-	+	-	-	-
8	+	+	-	-	-	-	-
9	+	+	-	+	-	-	-
10	+	+	-	+	-	-	-
11	+	+	-	+	-	-	-
12	+	+	-	+	-	-	-
13	+	+	-	+	-	-	-
14	+	+	-	+	-	-	-
15	+	+	-	+	-	-	-
16	+	+	-	+	-	-	-
17	+	+	-	+	+	-	-
18	+	+	-	+	-	-	-
19	+	+	-	+	-	-	-
20	+	+	-	+	-	-	-
21	+	+	-	-	-	-	-
22	+	+	-	+	-	-	-
23	+	+	-	+	-	-	-
24	+	-	-	-	-	-	-
25	+	+	-	-	-	-	-
26	+	-	-	-	-	-	-
27	+	+	-	+	-	-	-
28	+	-	-	-	-	-	-
29	+	+	-	+	-	-	-
30	+	+	-	+	+	-	-
31	+	-	-	-	-	-	-
32	+	+	-	+	-	-	-
33	+	-	-	-	-	-	-
34	+	+	-	+	-	-	-
35	+	+	-	+	-	-	-
36	+	-	-	-	-	-	-
EBV sero+ve (n=36)	36	28	0	23	3	0	0
EBV sero-ve (n=3)	3	0	0	0	0	0	0

Figure 4-23. RT-PCR on PBM RNA from normal EBV seropositives

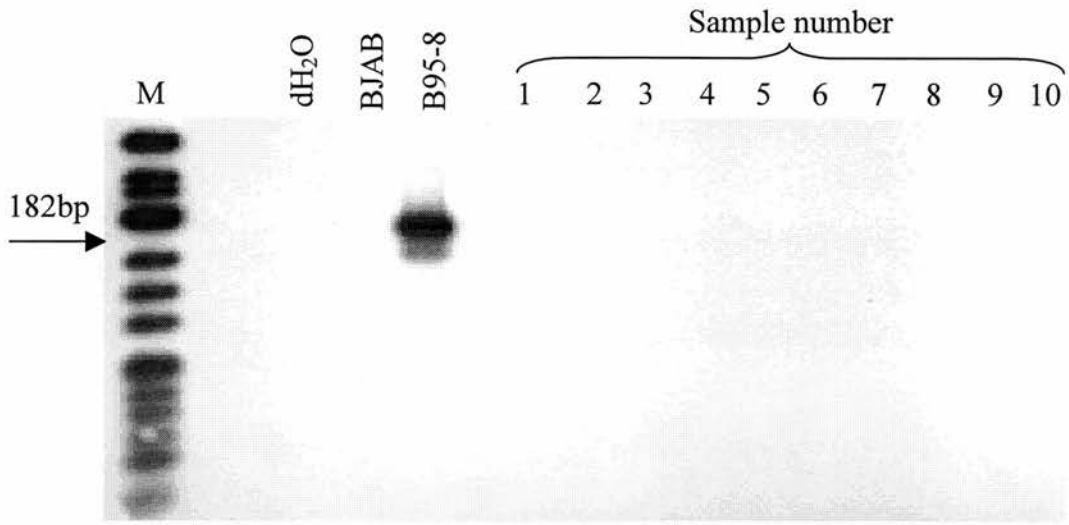
4-23a. β-actin PCR



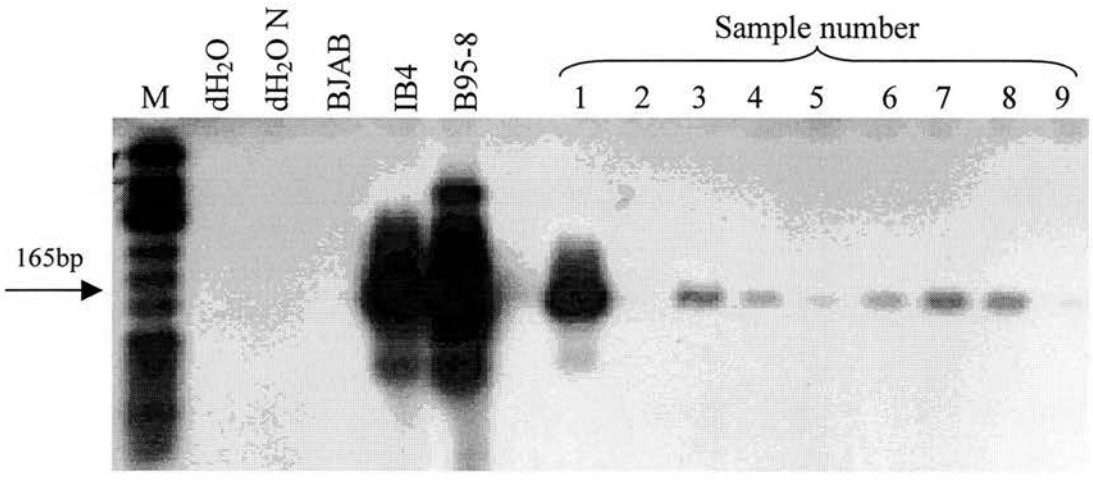
4-23b. EBERs PCR



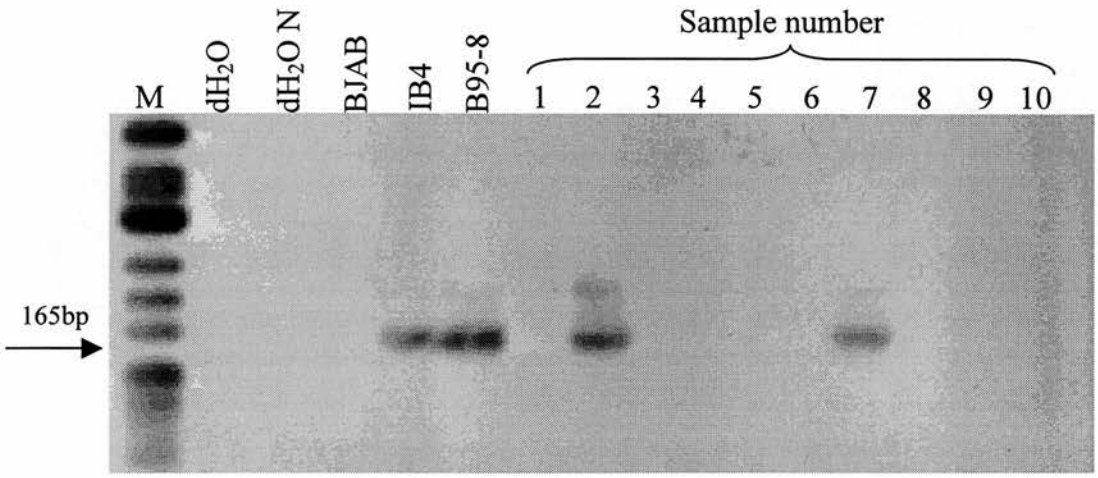
4-23c. LMP1 nested PCR



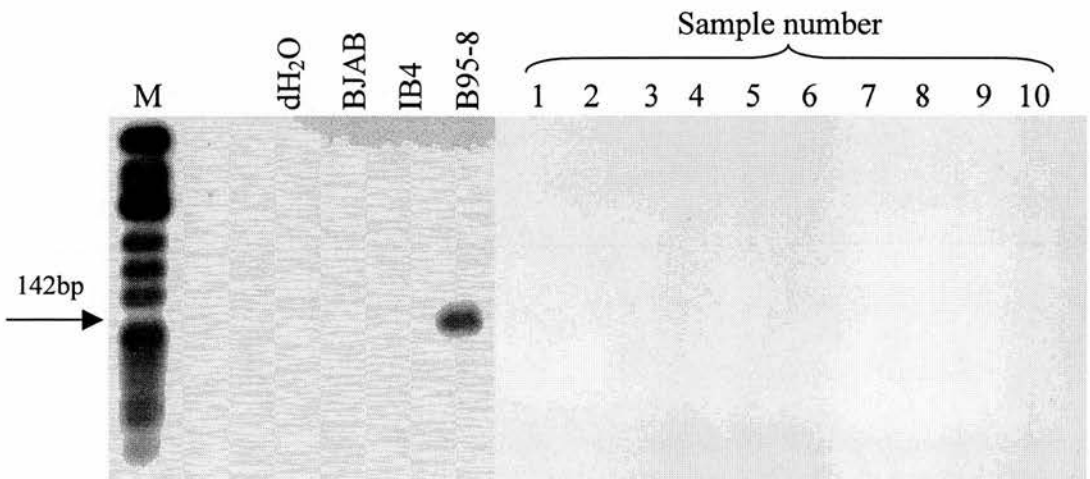
4-23d. LMP2a nested PCR



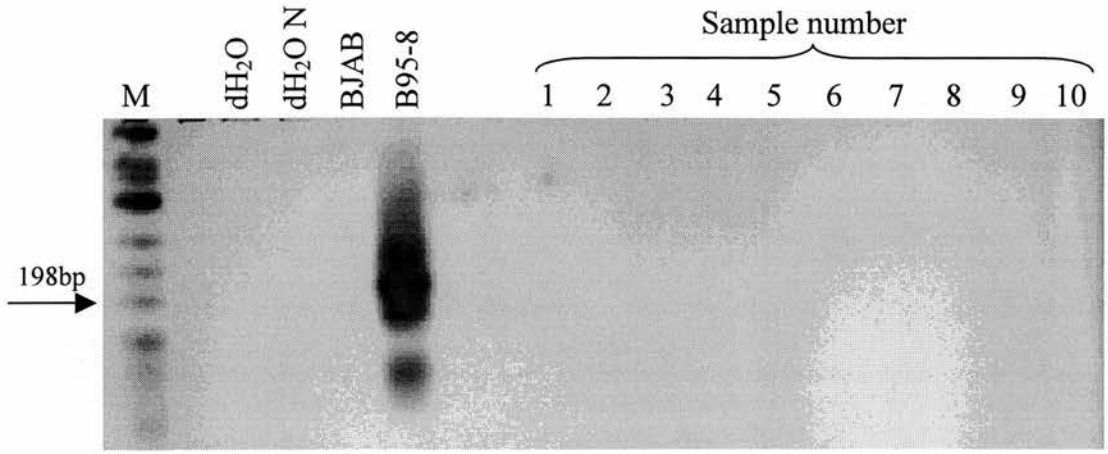
4-23e. LMP2b nested PCR



4-23f. gp350 PCR



4-23g. EBNA3c PCR



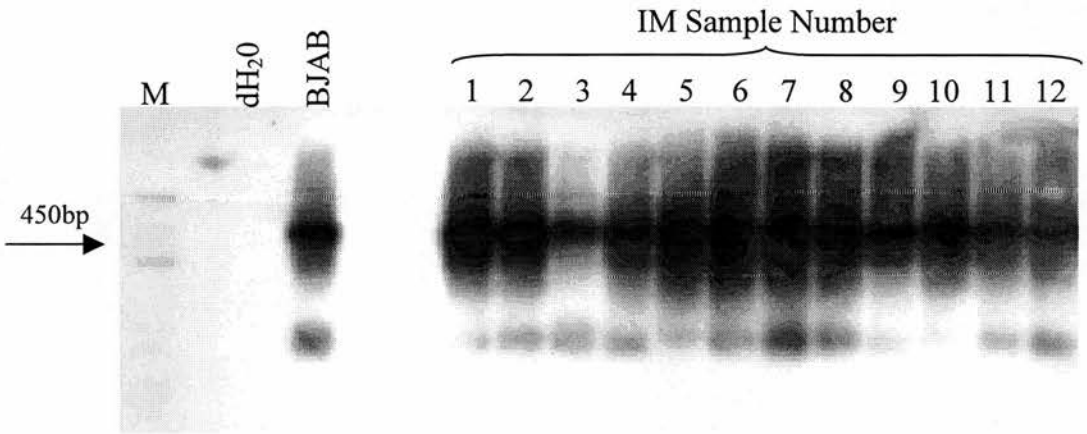
4.2.3 EBV gene expression in infectious mononucleosis (IM)

RT-PCR was carried out on cDNA from PBMs from 40 patients with acute IM to determine the pattern of EBV gene expression in these individuals. Autoradiographs of PCRs on representative samples for each transcript are shown in Figure 4-24a to g and data for all patients with IM is summarised in Table 4-15a and b.

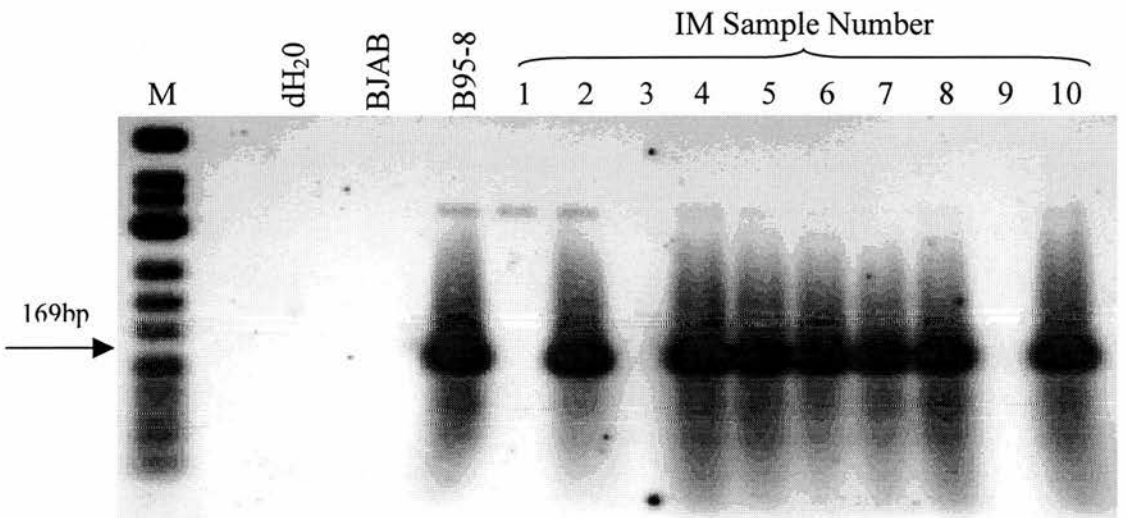
EBERs encoding transcripts were detected in 19 of 28 human β-actin positive IM samples tested patients (67.9%). EBERs were the only EBV transcripts detected in 6 patients (21.4%). LMP2a was detected in 1 patient and LMP2a with LMP2b detected in another individual. EBNA3c transcripts were detected in 2 patients (7%) and EBNA3c with gp350 was detected in 4 patients (14.3%). In 5 patients (18%), gp350 was detected in the absence of EBNA3c mRNA. These combinations of EBV gene expression contrast with the pattern of EBV gene expression detected in normal EBV positive individuals, where no LMP1, EBNA3c or gp350-encoding transcripts were detected.

Figure 4-24. RT-PCR on RNA from PBMs from patients with IM

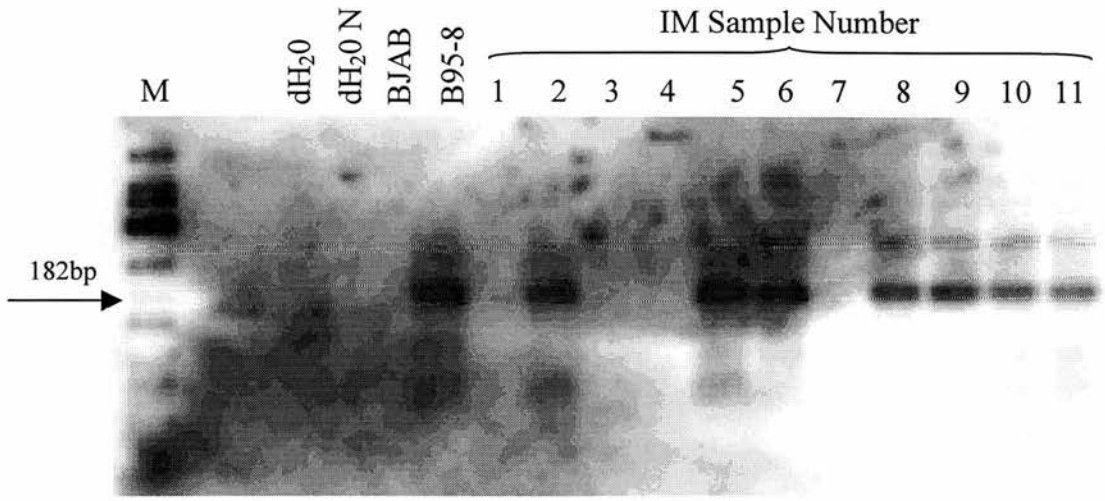
4-24a. β-actin PCR



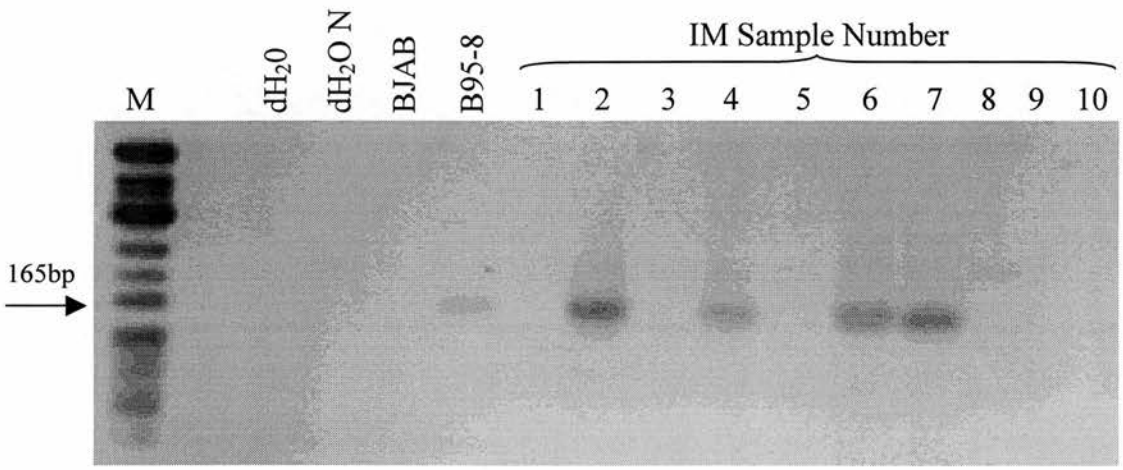
4-24b. EBERs PCR



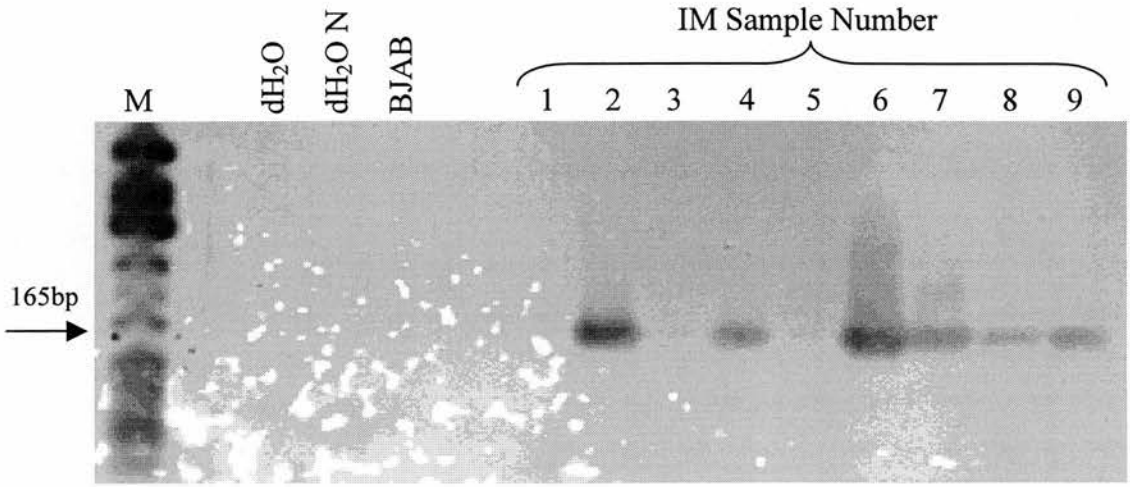
4-24c. LMP1 nested PCR



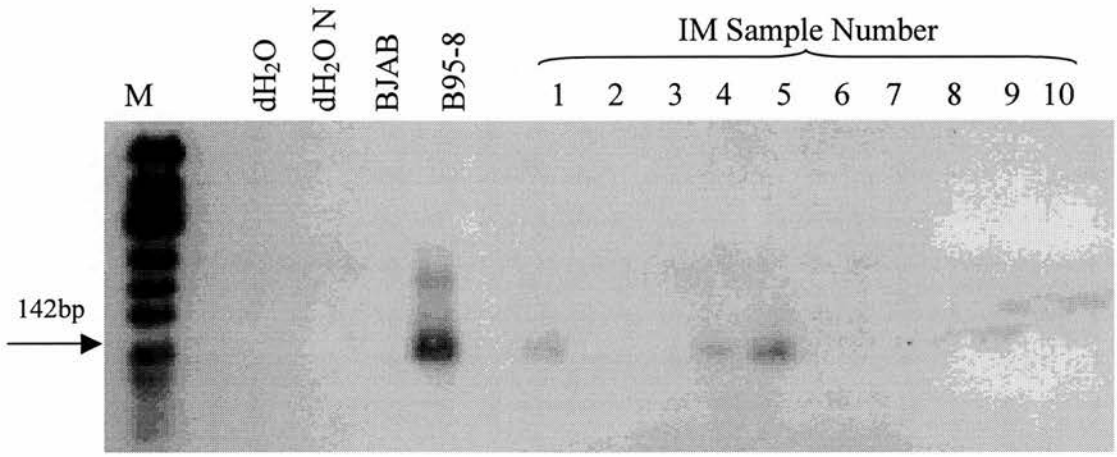
4-24d. LMP2a nested PCR



4-24e. LMP2b nested PCR



4-24f. gp350 PCR



4-24g. EBNA3c nested PCR

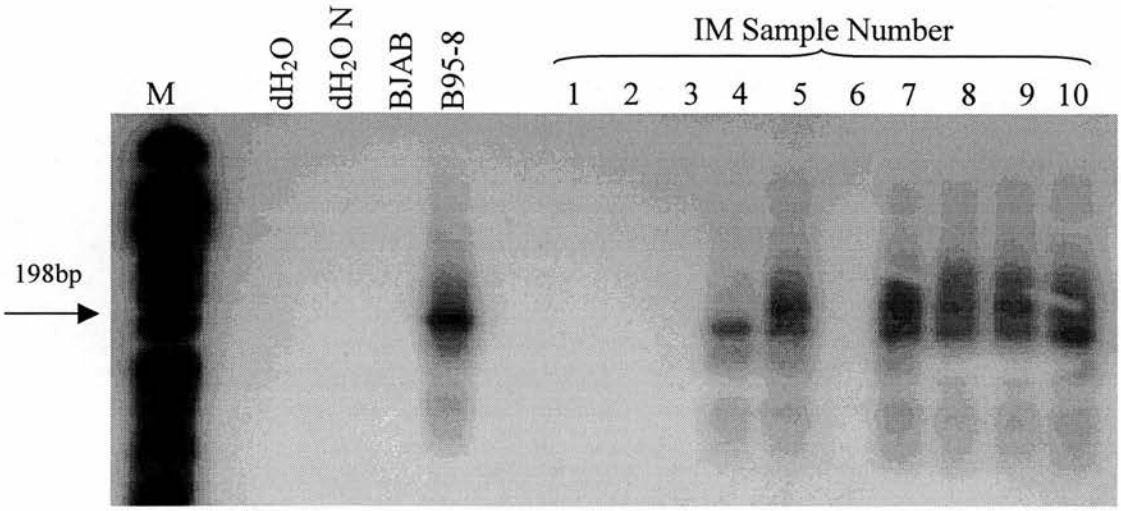


Table 4-15a. Summary of EBV gene expression in patients with IM

	EBV transcription pattern				
	Restricted	**Restricted + lytic**	**Latency III**	**Latency III + lytic**	**LMP1 (Lat II)**
Number of patients (n=28)	16	5	2	4	1

Table 4-15b. EBV gene expression in individual patients with IM

	EBV Transcript						
Patient No.	Actin	EBER1	LMP1	LMP2a	LMP2b	gp350	EBNA3c
1	+	+	-	+	-	+	-
2	+	+	-	-	-	-	-
3	+	+	-	+	-	-	-
4	+	+	-	+	+	+	+
5	+	+	-	-	-	-	-
6	+	-	-	-	-	-	-
7	+	+	-	-	-	+	-
8	+	+	+	+	+	+	+
9	+	-	-	-	-	-	-
10	+	+	-	-	-	-	-
11	+	-	-	-	-	-	-
12	+	-	-	-	-	-	-
13	+	-	-	-	-	-	-
14	+	-	-	-	-	-	-
15	+	+	-	-	-	-	-
16	+	-	-	-	-	-	-
17	+	-	-	-	-	-	-
18	+	+	-	-	-	-	-
19	+	+	+	-	-	-	-
20	+	+	-	-	-	-	-
21	+	+	+	+	+	+	+
22	+	+	-	-	-	+	-
23	+	+	+	+	-	+	-
24	+	-	-	-	-	-	-
25	+	+	+	+	+	-	+
26	+	+	-	+	+	+	-
27	+	+	-	+	+	+	+
28	+	+	-	+	+	-	+
Totals (%)	**28**	**19 (67.9% of actin +ves)**	**5 (18%)**	**10 (36%)**	**7 (25%)**	**9 (32%)**	**6 (21%)**

4.2.4 EBV gene expression in PTLD tumours, PBMs from cases of PTLD and SCID passaged tumour material

RNA was extracted from PBMs from eleven transplant recipients with PTLD and subjected to RT-PCR to detect human β-actin and EBV encoded transcripts. RNA was extracted from tumour biopsy material from 6 patients with PTLD and PBMs were also available from 3 of these patients. Tumour material from 5 patients had been passaged in SCID mice (carried out by Dr I. Johannessen). Patient details are shown in Table 4-16.

RT-PCR for detection of EBV encoded transcripts was performed on β-Actin positive samples to determine the pattern of EBV gene expression. Autoradiographs of PCRs for each transcript in representative samples are shown in Figure 4-25 and data for all samples is summarised in Table 4-16.

In all six PTLD biopsy samples, EBV gene expression was equivalent to that in LCLs, with detection of Latency III-like transcription (EBERs, EBNA3c, LMP1 and LMP2a and b) in addition to lytic replication (gp350). In tumours passaged in SCID mice the same pattern of EBV gene expression was detected as in the biopsy from which it was derived; that is latency III and lytic replication in all cases.

EBERs transcripts were detected in all 11 PBM samples from transplant recipients with PTLD, but the pattern of EBV gene expression varied in different patients (Table 4-16). In 10 of 11 samples (91%) LMP1, EBNA3c or gp350 transcripts were detected, transcripts which were not detected in normal healthy EBV carriers. In PBMs from 5 patients with PTLD (patients 3, 6, 10, 14 and 15) EBNA3c and gp350 transcripts were detected (latency III transcription with lytic replication). The other 6 patients had more restricted patterns of EBV transcription with LMP1 only in 2 cases and LMP1 with lytic replication, and in 1 case each; LMP2a only, LMP1 and LMP2a, and lytic replication with EBERs.

Where both PBMs and biopsy material were available (patients 1, 3 and 6) two patients (patients 3 and 6) had identical EBV gene expression in PBMs and biopsy material (Latency III plus lytic replication) but in patient 1 only LMP1 and

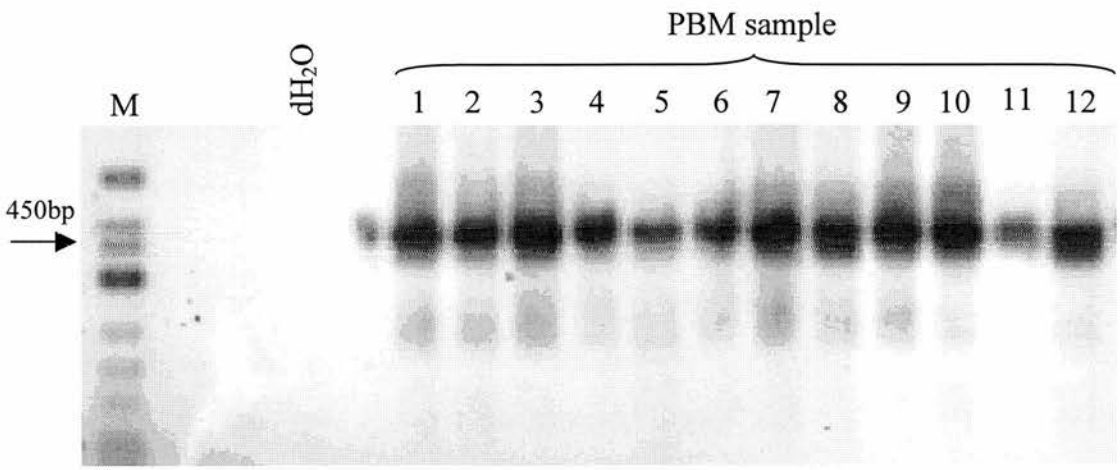
gp350 were detected in PBMs while latency III and lytic replication was detected in the corresponding biopsy material.

These results demonstrate that in PBMs from patients with PTLD, EBV genes are expressed which are not detected in normal healthy EBV carriers (that is, LMP1, EBNA3c and lytic replication).

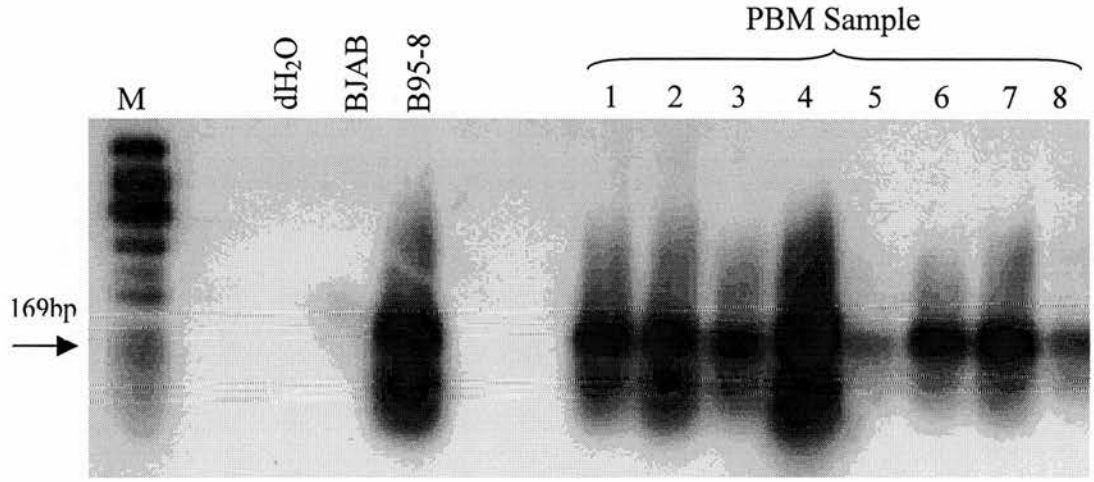
In 6 patients both EBV genome copy number and EBV gene expression data was available (patients 1, 3, 6, 9, 10 and 11). In 3 patients (patients 3, 6 and 10) with Latency III plus lytic replication in RNA from PBMs, EBV copy number was 3.3 x 10^3, 5 x 10^4 and 1 x 10^4 EBV genomes/10^6 PBMs respectively. In 2 patients with LMP1 detected in PBMs (9 and 12), the EBV genome copy number was 400 and 1250 genomes/10^6 PBMs respectively and in patient 1, in whom LMP1 and lytic replication was detected, the EBV copy number was 2.7 x 10^4 genomes/10^6 PBMs. The number of patients in this group is insufficient for statistical analysis, but it appears that detection of Latency III and lytic replication in PBMs is associated with high EBV load.

Figure 4-25. RT-PCR of RNA from PBMs from patients with PTLD

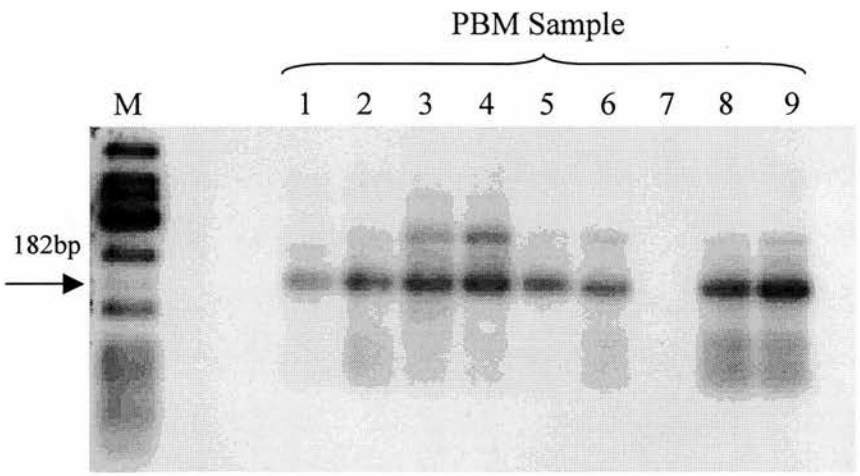
4-25a. β-actin PCR



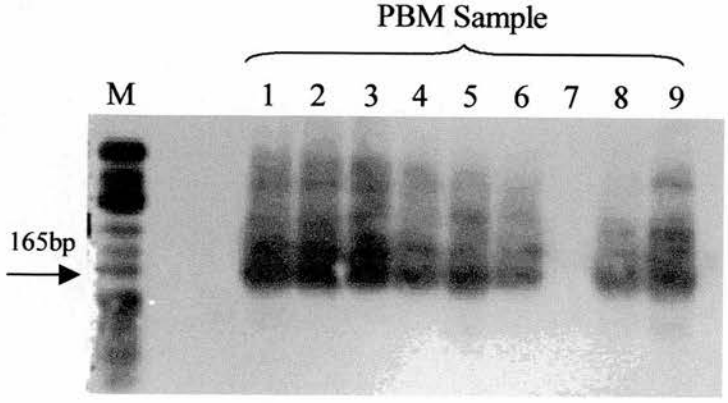
4-25b. EBERs PCR



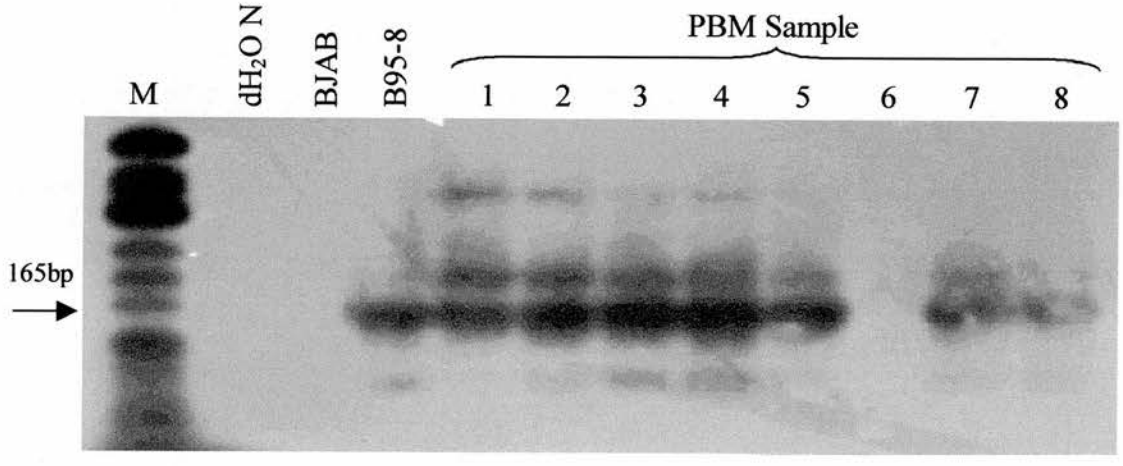
4-25c. LMP1 nested PCR



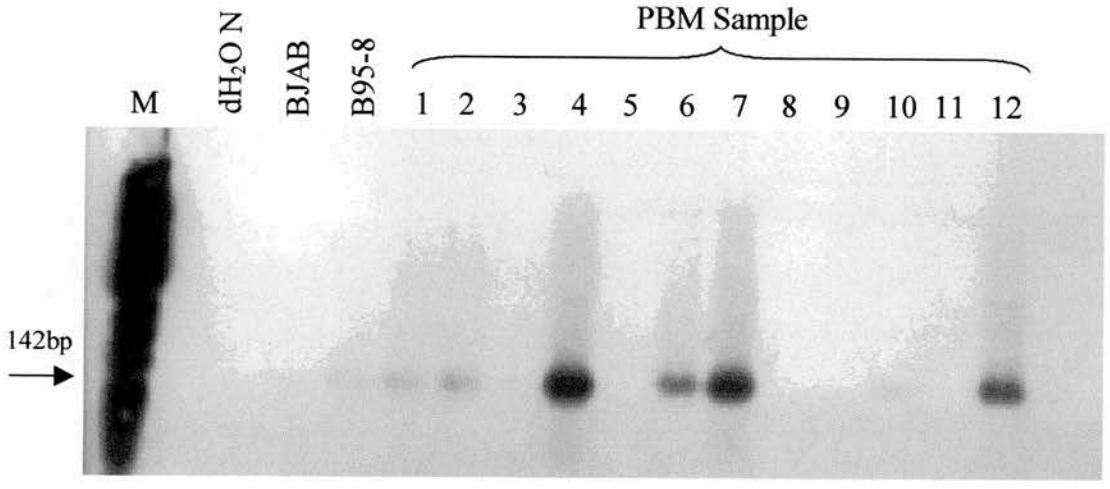
4-25d. LMP2a nested PCR



4-25e. LMP2b nested PCR



4-25f. gp350 PCR



4-25g. EBNA3c nested PCR

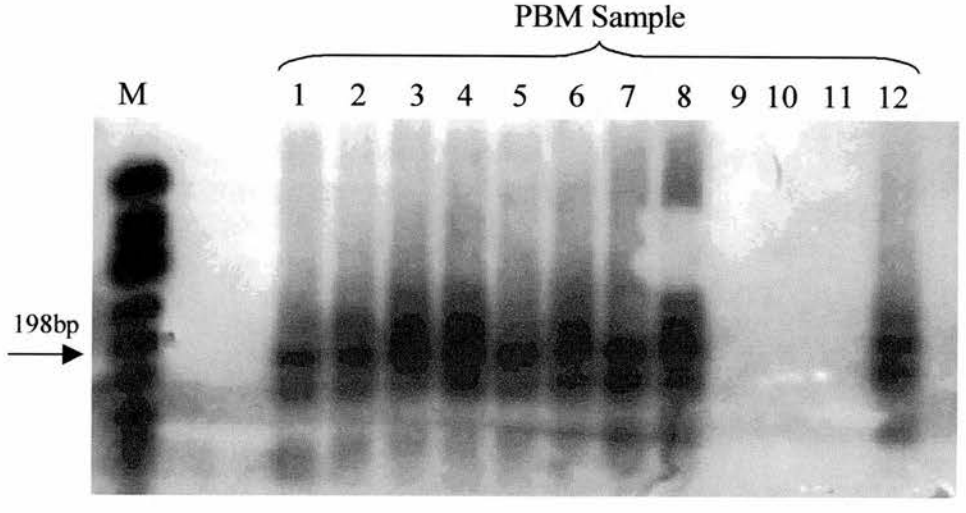


Table 4-16. Clinical details and EBV gene expression in patients with PTLD

Patient	Sample	Transplant Type	PTLD diagnosis	EBV Copy number	Actin	EBER1	LMP1	LMP2A	LMP2B	gp350	EBNA3C
1	PBMs	Renal	MM	27 000	+	+	+	-	-	+	-
	Biopsy				+	+	+	+	+	+	+
	SCID				+	+	+	+	+	+	+
2	Biopsy	Liver	IB	n/a	+	+	+	+	+	+	+
	SCID				+	+	+	+	+	+	+
3	PBMs	Liver	BLPD	3 300	+	+	+	+	+	+	+
	Biopsy				+	+	+	+	+	+	+
	SCID				+	+	+	+	+	+	+
4	Biopsy	Heart	IB	n/a	+	+	+	+	+	+	+
	SCID				+	+	+	+	+	+	+
5	Biopsy	Heart	HD	n/a	+	+	+	+	+	+	+
	SCID				+	+	+	+	+	+	+
6	PBMs	Kidney	IB	50 000	+	+	+	+	+	+	+
	Biopsy				+	+	+	+	+	+	+

Patient	Sample	Transplant Type	PTLD diagnosis	EBV Copy number	Actin	EBER1	LMP1	LMP2A	LMP2B	gp350	EBNA3C
9	PBMs	Kidney	G-LN	400	+	+	+	-	-	-	-
10	PBMs	Kidney	IB	10 000	+	+	+	+	+	+	+
11	PBMs	Kidney	IB	n/a	+	+	-	+	-	-	-
12	PBMs	Kidney	IB	1 250	+	+	+	-	-	-	-
13	PBMs	Kidney	IB	n/a	+	+	+	+	-	-	-
14	PBMs	Kidney	IB	3 500	+	+	+	+	+	+	+
15	PBMs	Kidney	IB	n/a	+	+	+	+	+	+	+
16	PBMs	Kidney	IB	n/a	+	+	-	-	-	+	-

Key to PTLD diagnosis:

MM: multiple myeloma. IB: immunoblastic lymphoma. HD: hodgkins disease. G-LN: granulomatous lymphadenopathy. PH: plasmacytic hyperplasia. n/a: not available

4.2.5 *EBV gene expression in PBMs from cardiothoracic transplant recipients*

Having shown that EBV load is significantly elevated post-transplant and in PTLD, experiments were carried out to determine whether the pattern of EBV gene expression in PBMs following transplantation could predict PTLD development. These results were compared with those from normal donors and patients with IM or PTLD.

cDNA generated from RNA extracted from PBMs was analysed by PCR to amplify transcripts from the human β-actin gene and the EBV genes EBER1, LMP1, LMP2a/b, gp350 and EBNA3c. Autoradiographs of PCRs on serial samples from a representative patient are shown in Figure 4-26a-g.

Figure 4-26a-g. RT-PCR on serial samples from an EBV seropositive cardiothoracic transplant recipient.

Sample numbers shown represent serial samples from a single transplant recipient. Sample 1 was taken pre-transplant, and subsequent samples were taken post-transplant.

Figure 4-26a. β-actin PCR

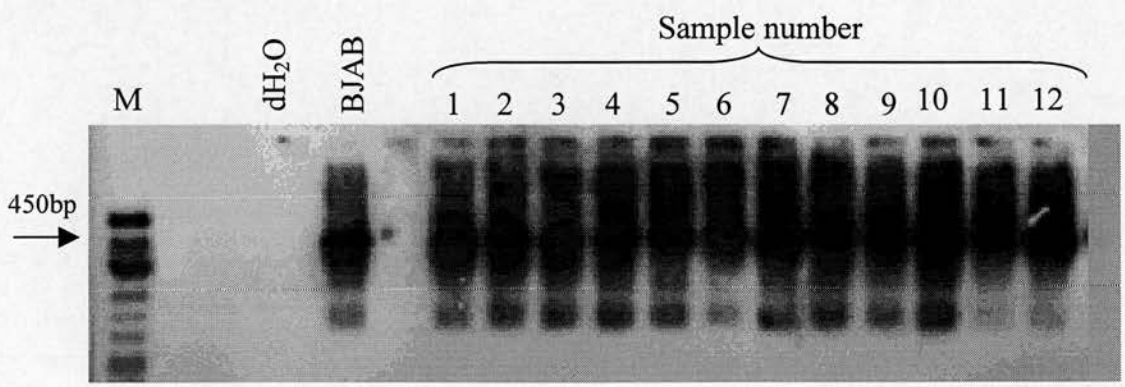


Figure 4-26b. EBER PCR

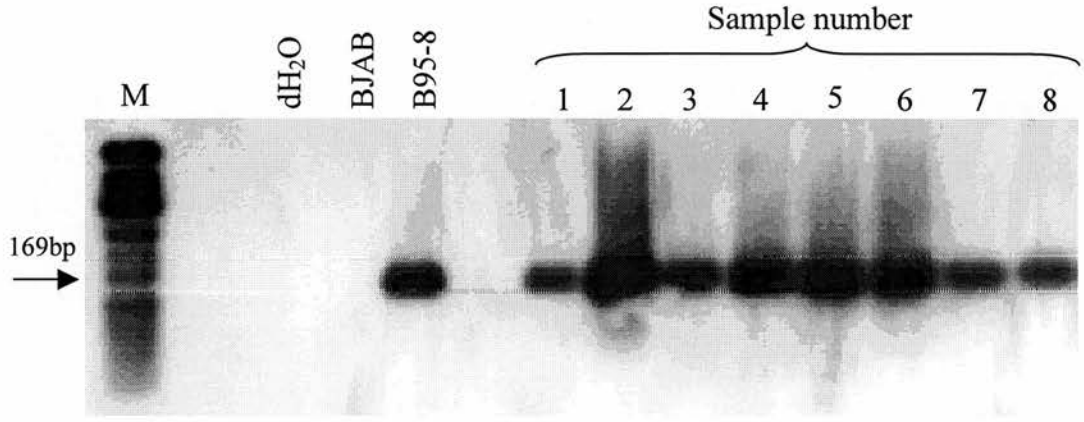


Figure 4-26c. LMP1 nested PCR

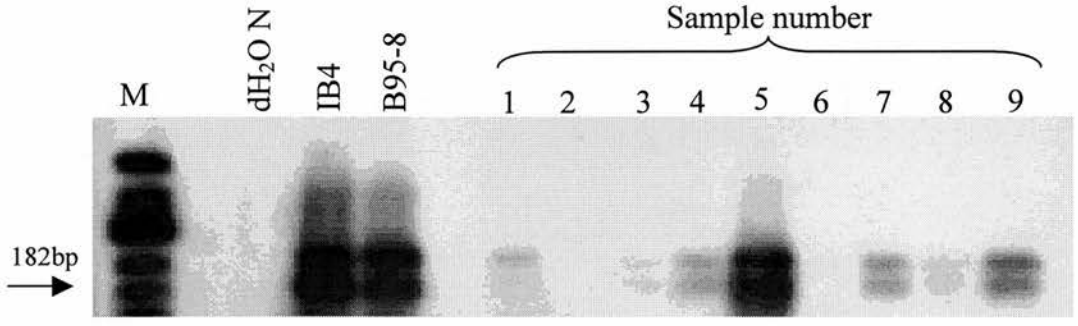


Figure 4-26d. LMP2a PCR

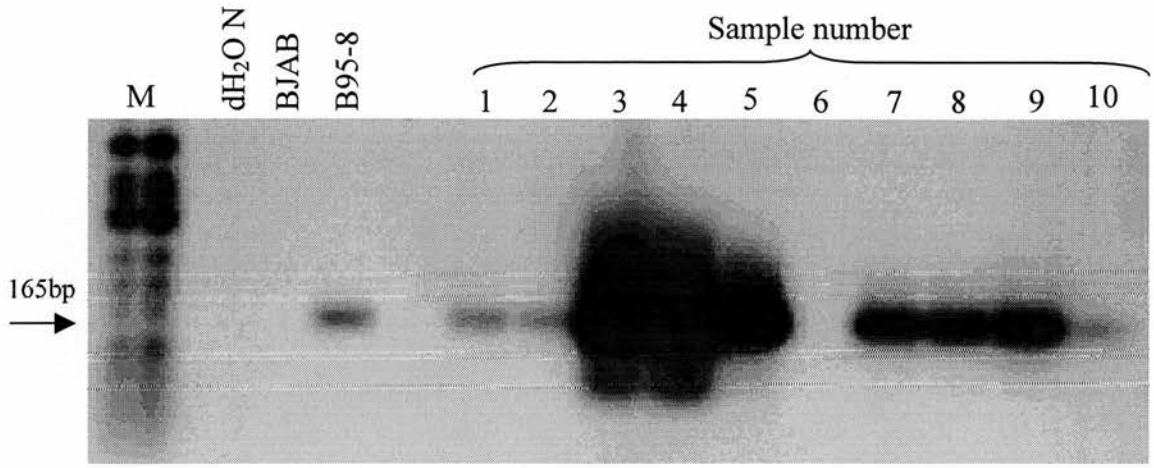


Figure 4-26e. LMP2b PCR

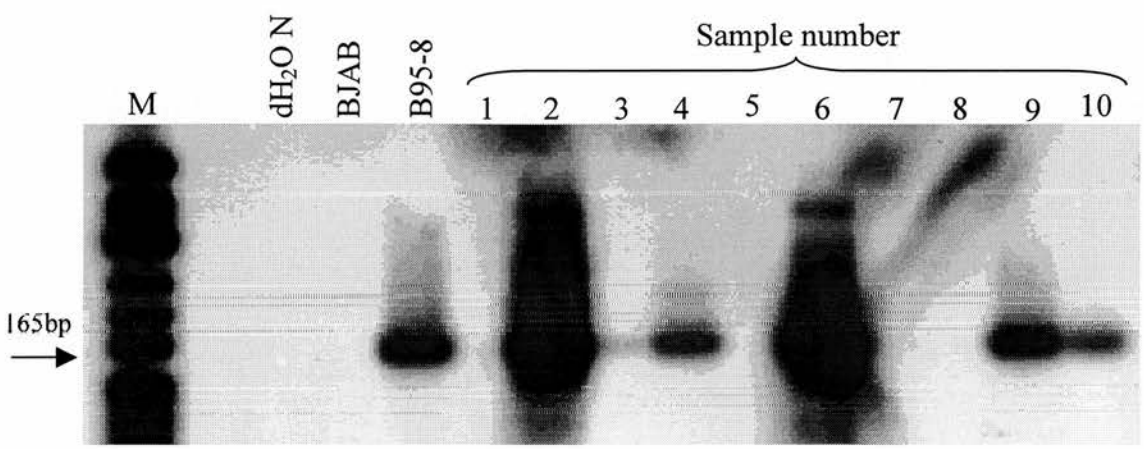


Figure 4-26f. EBNA3c PCR

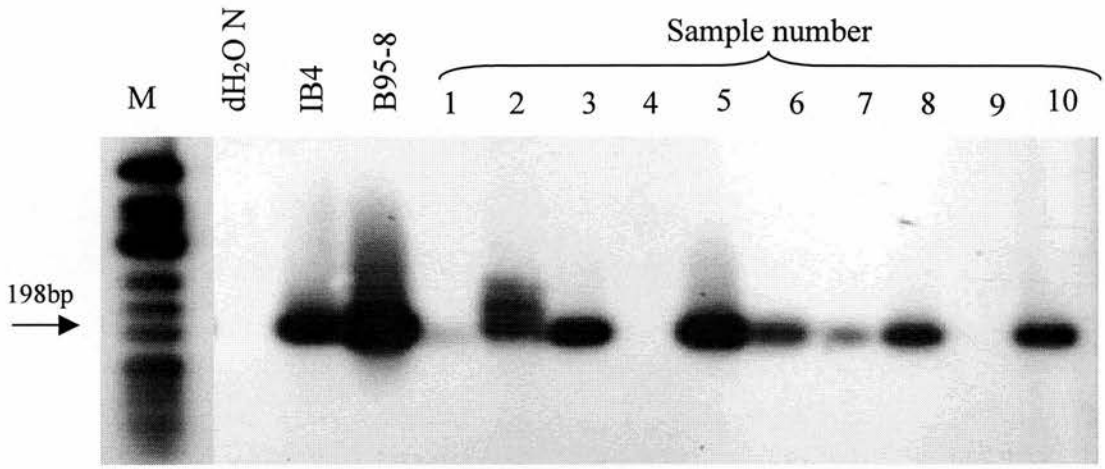
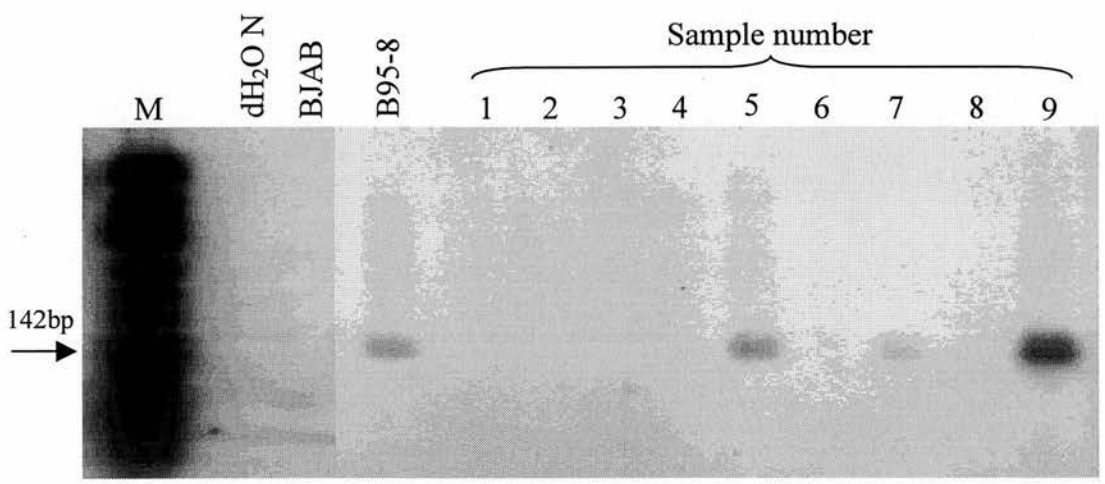


Figure 4-26g. gp350 PCR



4.2.5.1 EBV gene expression in pre-transplant samples

Pre-transplant samples from 95 patients were initially screened for the presence of amplifiable RNA using RT-PCR to detect human β-actin transcripts. β-actin RNA was detected in 77 of 95 samples (81.1%) and these samples were then tested for EBERs mRNA. Since this highly expressed transcript is expected to be present in all EBV infected cells, EBERs negative samples were eliminated from further analysis. This approach was justified by the previous analysis of EBV transcription in patients with IM (Table 4-15) in which LMP1, LMP2a/ b, gp350 and EBNA3c EBV transcripts were only detected in EBERs positive samples. Examples of autoradiographs obtained following PCR to detect EBV transcripts in serial samples from a representative EBV seropositive patient are shown in Figures 4-26a to g. RT-PCR results for all patients are summarised in Table 4-17.

EBERs mRNA was detected in 47 of 77 β-actin positive pre-transplant samples screened (61%) and these samples were subsequently tested for the EBV encoded latent transcripts LMP1, LMP2a, LMP2b and EBNA3c and the lytic transcript gp350. The combination of EBV transcripts detected in each sample was summarized according to previously described patterns of EBV gene expression *in vitro* and *in vivo*. Five patterns of EBV gene expression were identified:

1. Restricted EBV transcription, equivalent to that in normal EBV carriers: that is EBERs only, LMP2a/b or no detectable EBV transcription.
2. LMP1 transcription with or without LMP2a/b
3. Restricted latent gene expression with lytic replication (gp350 with no detectable EBNA3c).
4. EBNA3c without detectable lytic replication (unrestricted latency)
5. EBNA3c with gp350 (Unrestricted LCL-like latent and lytic transcription)

The patterns of EBV gene expression detected pre-transplant are shown in Table 4-17a. Restricted EBV transcription equivalent to that in normal healthy EBV carriers was detected in pre-transplant PBM samples from 64 of 77 patients (83%). Pre-transplant samples from 13 patients (17%) contained additional EBV transcripts

including EBNA3c in 2 patients (2.6%), EBNA3c and gp350 in 1 patient (1.3%), gp350 in the absence of EBNA3c in 2 patients (2.6%) and LMP1 in 8 patients (10.4%). Pre-transplant diagnosis of patients in whom additional transcripts were detected is shown in Table 4-17b.

Table 4-17a. EBV gene expression patterns pre- and post-transplant in cardiothoracic transplant recipients

	EBV transcription pattern Number (**%** of total for time point)					
Time of sample	**Restricted (EBERs, LMP2a/b)**	**LMP1 without EBNA3c**	**EBNA3c**	**Restricted latency + gp350**	**EBNA3c + gp350**	**Total**
Pre-Tx.	64 (**83**)	8 (**10**)	2 (**3**)	2 (**3**)	1 (**1**)	77
1st month post-tx.	26 (**65**)	4 (**10**)	2 (**5**)	7 (**18**)	1 (**3**)	40
1-6 months	101 (**79**)	11 (**9**)	7 (**6**)	7 (**6**)	2 (**2**)	128
6-18 months	47 (**66**)	10 (**14**)	1 (**1**)	9 (**18**)	4 (**6**)	71
18-30 months	31 (**63**)	4 (**8**)	7 (**14**)	4 (**8**)	3 (**6**)	49
30-42 months	8 (**47**)	4 (**24**)	2 (**12**)	2 (**12**)	1 (**6**)	17
Total	277	41	21	31	12	**382**

The table shows the number and % of PBM samples in which each pattern of EBV gene expression was detected during the time periods indicated.

Table 4-17b. Pre-operative diagnosis and transplanted organ in patients with unrestricted EBV gene expression pre-transplant.

	Transplant Type				
Pre-Tx gene expression	**H**	**L**	**H/L**	**Can-celled.**	**Pre transplant diagnosis (number)**
LMP1 (n=8)	4	2	1	1	DCM (2), ICM (1), Post-partum CM (1), eisenmengers disease (1), fibrosing alveolitis (1), emphysema + IHD (1), Tx Cancelled (1)
Restricted latency + gp350 (n=2)	1	1			Emphysema (1) Previous mitral valve replacement + coronary artery bypass graft (1)
Latency III (n=2)	2				ICM (1) Post-partum CM (1)
Lat III + gp350		1			COAD

CM: Cardiomyopathy. DCM: Dilated CM. ICM: Ischemic CM. IHD: Ischemic heart disease. COAD: Chronic obstructive arterial disease.

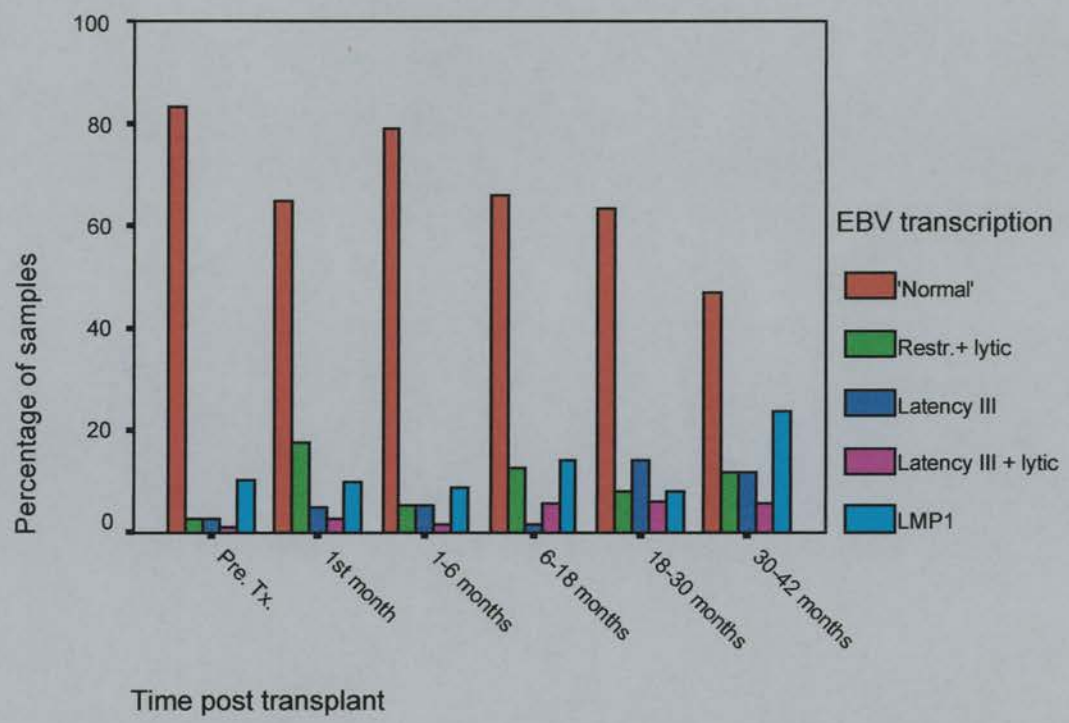
4.2.5.2 EBV gene expression in post-transplant samples

β-actin transcription was detected in RNA from 382 of 488 post-transplant PBM samples tested (78.3%) from 90 transplant recipients. EBERs transcripts were detected in 187 samples (49% of β-actin positive samples), which were then tested for the EBV encoded latent transcripts LMP1, LMP2a, LMP2b and EBNA3c and the lytic transcript gp350 (Results shown in Table 4-17). Additional EBV transcripts to those detected in healthy EBV seropositive donors were detected in 46 transplant recipients (51%). EBNA3c mRNA was detected post-transplant in 15 patients (16.7%) and lytic replication was also detected in 5 of these patients (5.6%). The patients in whom EBNA3c was detected were 9 males, 7 females aged 17 to 62 (mean 41.5 years). There was no association between detection of EBNA3c transcripts and patient age, sex or transplant type (p=0.126, 0.739 and 0.297 respectively, using Spearman's correlation). Lytic replication was detected with restricted latent gene expression in 23 patients (25.6%). There was no association between detection of gp350 and patient age, sex or transplant type (p=0.716, 0.334 and 0.075 respectively by Spearman's correlation). In 14 patients (15.6%) the only additional EBV transcript to be detected post-transplant, other than EBERs or LMP2 a/b, was LMP1. EBNA3c, LMP1 and gp350 mRNAs are not detectable in PBMs from normal donors, but are associated with EBV-associated disease (PTLD and IM). These EBV transcripts are also detectable pre- and post-transplant in transplant patients who show no signs of PTLD.

Samples were grouped according to the time post-transplant at which they were taken, that is the first month post-transplant, up to 6 months post-transplant, and subsequent 12 month periods: 6-18 months, 18-30 months, 30-42 months post-transplant. A summary of EBV transcription at each time point is shown in Table 4-17 and a bar chart showing the percentage of samples expressing each pattern of EBV gene expression during each time point is shown in Figure 4-27. There was a significant increase in detection of lytic replication with restricted latent gene expression from 3% pre-transplant to 18% in the first month post-transplant (p=0.028). This increase in lytic transcription was detected despite routine use of prophylactic low-dose acyclovir in the first three months post-transplant. In the

subsequent period up to 6 months post-transplant, the distribution of EBV transcription patterns returns to pre-transplant levels (83% pre-transplant, 79% in 1-6 months post-transplant, p=0.462). With increasing follow-up time, there is a steady decrease in the proportion of samples with a 'normal' pattern of EBV gene expression and a corresponding increase in the proportion of samples with detectable lytic replication (with and without EBNA3c), and in the proportion of samples in which more unrestricted latency was detected (EBNA3c and LMP1). The proportion of samples in which additional EBV transcripts were detected becomes significantly different from pre-transplant levels by 30 months post-transplant (p=0.002).

Figure 4-27. EBV gene expression in PBMs at different time points following cardiothoracic transplantation



Graph shows the percentage of samples expressing different EBV gene expression patterns at each time point pre- and post-transplant.

EBV transcription patterns:
Summarised EBV transcription patterns are based upon detection of the following transcripts:
'Normal': Restricted EBV gene expression, equivalent to normal EBV carriers (EBERs only, LMP2a/b or no detected EBV transcripts).
Restr. + lytic: Restricted latency (no EBNA3c) with gp350.
Latency III: EBNA3c.
Latency III + lytic: EBNA3c and gp350.
LMP1: LMP1 in the absence of EBNA3c (Latency II)

Results showing pre-transplant EBV gene expression for all patients and corresponding post-transplant changes are shown in Table 4-18. Thirty-four of 77 transplant recipients (44.2%) with restricted pre-transplant EBV gene expression had additional EBV transcripts (LMP1, EBNA3c or gp350) detected in one or more post-transplant samples. Thirty-five patients (45.5%) with normal EBV transcription pre-transplant did not have detectable levels of additional EBV transcripts in post-transplant samples. Only pre-transplant samples were available from 8 patients.

Of 13 transplant recipients with LMP1, EBNA3c or gp350 mRNAs detected pre-transplant, 6 patients continued to express additional EBV transcripts post-transplant, whereas EBV gene expression in post-transplant samples from 5 patients was equivalent to normal carriers.

Table 4-18. Post-transplant changes in EBV gene expression

		Pre-transplant EBV gene expression				
		Restricted (n=69)	EBNA3c (n=2)	Restricted + Lytic (n=2)	EBNA3c + lytic (n=1)	LMP1 (n=8)
Post-transplant EBV gene expression						
Restricted EBV gene expression in all post-transplant samples		35			1	4
Additional EBV transcripts detected post-tx.	EBNA3c	5	1			
	EBNA3c + gp350	3	1			
	Restricted latency + lytic	17		1		
	LMP1	9				

Table shows a summary of post-transplant changes in patients separated on the basis of pre-transplant EBV gene expression. Numbers of patients with each pattern of EBV gene expression pre-transplant is shown across the top of the table, and the pattern of gene expression detected post-transplant in the corresponding group is shown at the side.

4.2.6 *Statistical analysis of EBV transcription*

4.2.6.1 Influence of immunosuppression on EBV transcription

Post-transplant EBV transcription was analysed in comparison to that detected in normal EBV carriers and was defined as remaining equivalent to normal EBV carriers or as containing additional transcripts. There was no association between detection of additional transcripts and transplant type or patient sex (p=0.270 and 0.689 respectively by Spearman's correlation).

The immunosuppressive regime initially given to all transplant recipients was decreasing doses of cyclosporin, azathioprine and prednisolone with treatment for rejection using methylprednisolone. Twelve patients (14.6%) had their principal immunosuppressive treatment switched from cyclosporin to FK506 during the follow-up period. Using the Spearman correlation there was no association between use of FK506 and change in EBV transcription pattern (p=0.066). CMV viremia was noted in 21 transplant recipients (25.6%) but there was no association between CMV viremia and EBV transcription pattern (p=0.229). There was also no significant difference in EBV transcription pattern (p=0.267) between patients who did or did not experience acute graft rejection episodes (acute rejection in 33 patients (40.2%)). These results are summarised with patient numbers for each group in Table 4-19.

Linear regression analysis was used to assess associations between detection of additional transcripts to normal donors (ie LMP1, EBNA3c and gp350), and treatment in the period immediately preceding sampling (Table 4-20). There was no significant association with occurrence of rejection episodes, whether assessed by noting rejection in the previous month from clinical notes, or dose of methylprednisolone received in the preceding 2 weeks, or preceding month. There was no association between dose of cyclosporin received in the preceding month and detection of EBV transcripts.

The influence of long term immunosuppression on EBV gene expression in the PBMs of transplant recipients was assessed by multivariate analysis (analysis of variance) (Table 4-21). The total cumulative level of immunosuppressive therapy

received prior to samples being taken was assessed in relation to detection alternative patterns of EBV gene expression. No significant differences were detected in the pattern of EBV transcription and cumulative dose of cyclosporin, prednisolone or methylprednisolone. Detection of LMP1 alone (latency II) was associated with significantly higher cumulative dose of azathioprine than that associated with other transcripts (p=0.003). Detection of EBNA3c (Latency III) is associated with high cumulative dose of FK506 (p=0.005). Use of FK506 therefore appears to be associated with unrestricted latent gene expression in transplant recipients.

Table 4-19. Changes in EBV transcription in patients under FK506 treatment or experiencing CMV viremia or rejection episodes

	EBV transcription pattern (number of patients)	
	Detection of additional EBV transcripts post-transplant	Restricted post-transplant
Factor (number of patients)		
FK506 (12) p= 0.066	10	2
Cyclosporin (70)	37	33
CMV + (21) p=0.229	14	7
CMV – (61)	33	28
Rejection (33) p= 0.267	20	13
No rejection (49)	27	22

Table shows the number of transplant recipients with altered EBV gene expression post-transplant separated on the basis of the drug regime used (FK506 or cyclosporin), or whether patients experienced CMV viremia or acute rejection episodes.

Table 4-20. Analysis of immunosuppression dose and rejection episodes in period immediately prior to sampling and influence on EBV transcription post-transplant.

Treatment in period preceding sample	Linear regression analysis *p value.*
Methylprednisolone dose in previous 2 weeks	.387
Methylprednisolone dose in previous month	.542
Rejection in previous month	.579
Mean daily cyclosporin dose in month preceding sample	.862

Linear regression analysis was used to determine whether detection of additional EBV transcripts to those in normal EBV carriers was associated with graft rejection or dose of immunosuppressive drugs in the period preceding the sample. No significant association was found between any of the parameters tested and detection of unrestricted EBV gene expression.

Table 4-21. Multivariate analysis of association between cumulative immunosuppressive drug dose and detection of EBV transcripts.

Table shows cumulative drug dose associated with detection of alternative patterns of EBV transcription in transplant recipients. Variation between groups was analysed by ANOVA, and multiple cross comparisons were made by Tukeys ANOVA. Significant differences in drug dose associated with particular transcription patterns are highlighted (*).

Drug		Drug dose			Significant comparisons (Tukeys ANOVA)
	Transcription pattern	Mean	Minimum	Maximum	
CSA	Restricted	44048.1	0	21138	none
	Rest. + lytic	52725.1	1291	166908	
	Latency III	77474.2	0	163441	
	Lat. III + lytic	84477.3	9084	172860.5	
	Lat. II (LMP1)	59353.5	0	208110	
AZA	Restricted	11690.2	0	121250	Lat II> Restricted P=0.003
	Rest. + lytic	20416.3	545	106975	
	Latency III	23501	280	96220	
	Lat. III + lytic	14435	11960	19385	
	Lat. II (LMP1)	26614*	150	114675	
Pred	Restricted	1735.6	0	6647.5	none
	Rest. + lytic	1795.0	225	7027.8	
	Latency III	1972.7	692.8	4254.6	
	Lat. III + lytic	2307.9	670	3573.8	
	Lat. II (LMP1)	1957	0	6247.5	
Mpred	Restricted	1101.2	100	5250	none
	Rest. + lytic	-	-	-	
	Latency III	3000	1250	4750	
	Lat. III + lytic	-	-	-	
	Lat. II (LMP1)	3089.3	500	9250	
FK506	Restricted	15776.1	52.3	108812.8	Lat III> Restricted (P=0.001) Restr + lytic (p=0.005) Lat III + lytic p=0.005 Lat II (LMP1) p=0.008
	Rest. + lytic	2394.4	2029.9	2758.9	
	Latency III	248829.3*	248430	249228.6	
	Lat. III + lytic	1254.8	1131.3	1377.9	
	Lat. II (LMP1)	52438.7	557.1	249977.9	

4.2.6.2 Association between EBV gene expression and EBV copy number in transplant recipients

In all normal healthy individuals and 83% of patients awaiting transplantation EBV gene expression is restricted and EBV genome copy number is low (<1 to 200 genomes/10^6 PBMs). Following transplantation EBV copy number increases to levels equivalent to cases of IM (35.4% of patients) or PTLD (12.5% of patients) and EBV transcripts are detected which are not present in normal donors, notably transcripts associated with unrestricted latency (EBNA3c) and lytic replication (gp350). Detection of these latent and lytic transcripts in transplant recipients without PTLD indicates that following transplantation there is a loss of restriction in EBV transcription, which may contribute to increased EBV copy number. EBV genome copy number in PBMs could increase post-transplant through lytic replication producing high numbers of EBV genomes in individual productively infected cells or proliferating EBV infected B-lymphocytes could lead to high numbers of latently infected cells in peripheral blood.

EBV copy number in samples displaying different combinations of EBV gene expression is shown in Table 4-22. Mann-Whitney U tests were used to assess the association between individual EBV transcripts and EBV copy number (Table 4-23). Using this test there was a highly significant correlation between increased EBV copy number and detection of all EBV transcripts when tested as separate variables ($p \leq 0.005$ in each case): EBERs, LMP2a, LMP2b, LMP1, gp350 and EBNA3c. This association may be interpreted as being due to more ready detection of the EBV transcripts with increased numbers of infected cells in samples with increased EBV load. A box-plot of EBV load in samples with different patterns of EBV gene expression is shown in Figure 4-28.

The highest EBV loads were found in samples in which latency III and lytic replication were detected together (20 to 1 x 10^5 genomes/10^6 PBMs, mean = 1.9 x 10^4 genomes/10^6 PBMs) (Figure 4-27) ($p<0.001$ compared to EBNA3c or LMP1, $p=0.004$ compared to restricted latency with lytic replication). EBV load in samples expressing lytic replication and restricted latent transcription (mean=2 x 10^3

genomes/10^6 PBMs) are not significantly higher than in samples expressing EBNA3c or LMP1 without lytic replication (p=0.103 and 0.142 respectively). A combination of unrestricted latent gene expression and lytic replication are therefore involved in increasing EBV copy number in PBMs of transplant recipients. Samples with EBNA3c (Latency III) without lytic replication have EBV loads equivalent to samples with LMP1 (p=0.604) but EBV load with both of these forms of latent gene expression are significantly higher than in samples with restricted latent gene expression (p=0.04 and <0.001 respectively). The combination of latency III and lytic replication is clearly associated with high EBV copy number following transplantation, and it is this pattern of EBV gene expression that is most commonly detected in biopsies and PBMs of patients with PTLD.

Table 4-22. EBV copy number and EBV transcription in post-transplant PBM samples.

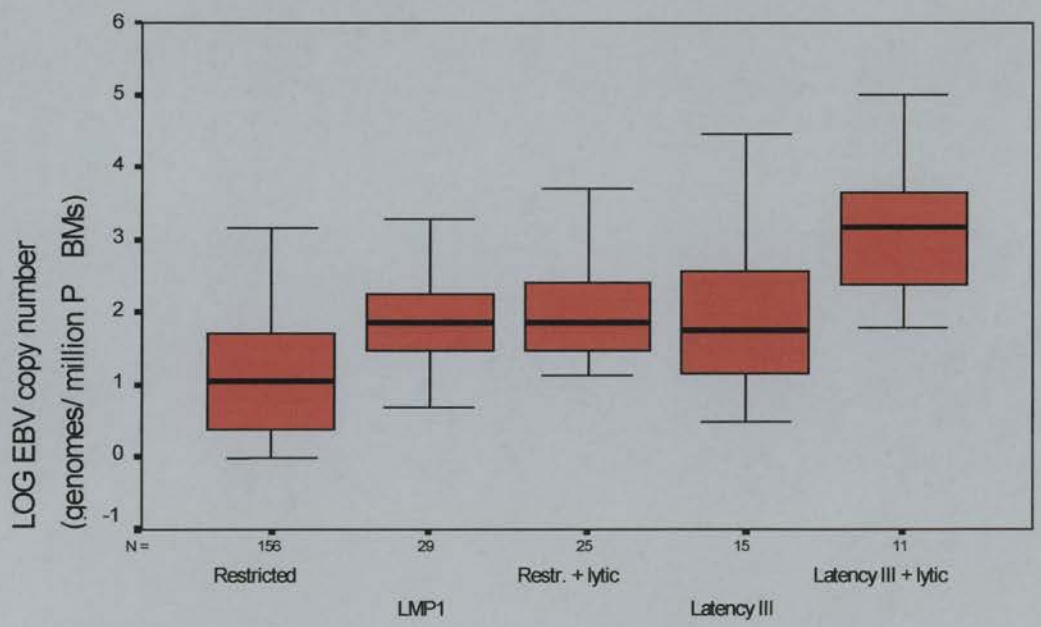
EBV transcription pattern	EBV copy number (genomes/ 10^6 PBMs)		
	Range	Median	Mean
No EBV mRNA detected	0-1.5 x 10^3	1	40
EBERs only	0-1 x 10^4	1	205
LMP2a/b only	0-1 x 10^4	20	276
LMP1 + LMP2a/ b	0-2 x 10^3	57	196
LMP1 only	0-90	50	42
Restricted latency + lytic replication (gp350)	0-7 x 10^3	60	766
Latency III (EBNA3c)	0-3 x 10^4	20	2.8 x 10^3
Latency III + lytic	60-1 x 10^5	1.5 x 10^3	1.95 x 10^4

Table 4-23. Association between individual EBV transcripts and EBV copy number.

	EBERs	LMP2a	LMP2b	LMP1	gp350	EBNA 3c
Mann-Whitney U test result	**P<0.001**	**P<0.001**	**P=0.001**	**P=0.001**	**P<0.001**	**P=0.005**

Mann-Whitney U tests were performed to analyze the association between expression of EBV transcripts and EBV copy number. There was a highly significant association between detection of all transcripts and increased EBV copy number.

Figure 4-28. Boxplot of EBV copy number in post-transplant PBM samples with different patterns of EBV gene expression.



Red boxes show the interquartile range and bold black horizontal lines show the median EBV copy number for samples displaying the pattern of EBV gene expression indicated .EBV load in samples with latency III with lytic replication are significantly higher than EBNA3c or LMP1 alone ($p<0.001$) and is significantly higher than samples with restricted latency with lytic replication ($p=0.004$). Samples expressing EBNA3c or LMP1 have significantly higher EBV load than in samples with restricted latency ($p=0.04$ & <0.001 respectively). EBV load in samples expressing EBNA3c, LMP1 and restricted transcription with lytic replication are not significantly different.

4.2.6.3 Comparison of EBV gene expression in PBMs from transplant recipients with normal donors and patients with IM and PTLD

The patterns of EBV gene expression detected in transplant recipients was compared with that detected in patients with IM, or PTLD and with normal healthy EBV seropositives. The numbers and percentage of samples from each group displaying each pattern of EBV gene expression are shown in Table 4-24. In all normal EBV carriers tested restricted EBV latency was detected. Restricted EBV latency, equivalent to that in normal EBV carriers was also detected in PBMs from 84% of patients awaiting transplantation, 57% of patients with IM and 27% of patients with PTLD. In 45.5% of patients with PTLD latency III with lytic replication can be detected in the peripheral blood whereas this pattern of EBV gene expression is only detected in 14% of patients with IM.

Levels of EBV DNA equivalent to that in patients with PTLD or IM were detected in transplant recipients without apparent disease. EBV gene expression in the corresponding samples from each patient is shown in Table 4-25. It is clear from this data that unrestricted EBV latent and lytic gene expression is associated with increased viral load in transplant recipients, although in some patients additional transcripts are detectable without accompanying high viral load. In all samples with high EBV load, equivalent to patients with PTLD (3500 - 1 x 10^6 genomes/10^6 PBMs), a less restricted pattern of EBV gene expression to that in normal donors was detected. Lytic replication was detected in 58% of samples with high EBV load, and lytic replication was detected together with EBNA3c in 33% of patients. Lytic replication was detected in 42% of patients with EBV loads equivalent to IM, but in 39% of IM patients, lytic replication was detected with restricted latent gene expression.

Eighty-two percent of patients with EBV loads equivalent to normal donors (0-40 genomes/10^6 PBMs) also have the same restricted EBV gene expression. However, additional transcripts to those detected in normal controls are present in patients whose EBV load remains low: lytic replication was detected in 3 patients, EBNA3c detected in 1 patient, and LMP1 in 2 patients.

Table 4-24. Summary of EBV gene expression in transplant recipients and controls

	EBV transcription pattern (number of samples and %)									
	Restricted latency[4]		Restricted latency[4] + lytic		Lat III (EBNA3c)		Lat III + lytic (EBNA3c + gp350)		LMP1 only	
Patient group	Count	%	Count	%	Count	%	Count	%	Count	%
pre tx[1].	64	83.8%	2	2.7%	2	2.7%	1	1.4%	8	9.5%
post. tx[2]	213	69.8%	29	9.5%	19	6.2%	11	3.6%	33	10.8%
Normals[3]	36	100.0%								
IM	16	57.1%	5	17.9%	2	7.1%	4	14.3%	1	3.6%
PTLD	3	27.3%	1	9.1%			5	45.5%	2	18.2%

1. Pre tx. : pre- transplant. 2. Post tx.: all post-transplant samples tested. 3. Normal healthy donors. 4. Restricted latency: EBV gene expression equivalent to healthy EBV seropositives (ie EBERS, LMP2a/b, or no detectable EBV transcripts)

Table 4-25. EBV gene expression in transplant recipients with EBV load equivalent to that in EBV-associated diseases and normal carriers

	Post-transplant EBV transcription pattern					
Patients with post-tx EBV load in range associated with EBV conditions (number)	Restricted latency	Restricted latency + gp350	EBNA3c	EBNA3c + lytic	LMP1	Not tested
Equivalent to PTLD[1] (n=12)	**0**	**3 (25%)**	**4 (33.3%)**	**4 (33.3%)**	**1 (8.3%)**	**0**
Equivalent to IM[2] (n=36)	**9 (25%)**	**14 (39%)**	**5 (13.9%)**	**1 (2.8%)**	**7 (19.4%)**	**0**
Equivalent to normal carriers[3] (n=50)	**28 (82.4%)**	**3 (8.8%)**	**1 (2.9%)**	**0**	**2 (5.9%)**	**16**
Total (%)	**37 (45.1%)**	**20 (24.4%)**	**10 (12.2%)**	**5 (6.1%)**	**10 (12.2%)**	

Interquartile range of EBV load in patient groups: 1. PTLD: 3500-1 x 10^6 genomes/10^6 PBMs. 2. IM: 40-1500 genomes/10^6 PBMs. Normal donors: 1-40 genomes/10^6 PBMs.

4.2.7 Predictive value of testing for EBV load and EBV gene expression in detecting PTLD

The effectiveness of a diagnostic test is dependent on a balance between the sensitivity and specificity of the test, since it should be able to correctly distinguish the disease state (specificity), and not produce false negative results (sensitivity). The intervention, which would follow a postive diagnostic test, is also a factor, in terms of whether the therapy is costly or harmful to the patient, in which case false positive tests are undesirable.

In the case of PTLD, whilst increased EBV load and detection of EBNA3C and lytic transcripts distinguish transplant recipients and patients with PTLD from normal donors, these factors are not specific indicators of PTLD development since they are also detected in some transplant recipients who do not develop disease. However since many early PTLDs respond to reduced immunosuppression, and regress without invasive or damaging treatments, diagnostic tests for PTLD do not need to be highly specific since it would not harm a patient to be treated on a precautionary principal, even if they would not have ultimately developed PTLD without treatment.

Therefore detection of high EBV loads (over 1500 genomes/10^6 PBMs, the upper level detected in IM) or detection of EBV transcripts not normally detected in healthy donors (such as EBNA3C or gp350) could be used as precautionary indicators to highlight patients who may be at risk of developing PTLD and immunosuppressive therapy could be modulated as appropriate. EBV load appears to be the most sensitive diagnostic test available for detecting development of PTLD, because all patients with PTLD had high EBV loads, but some patients with PTLD did not have detectable EBNA3C or gp350 transcripts in PBMs. Therefore it is less likely that a patient developing PTLD would be missed by routine quantitative PCR testing as opposed to RT-PCR for EBV mRNAs.

5 Discussion

5.1 Introduction

This study set out to investigate the course of EBV infection following cardiothoracic transplantation in order to examine whether virological factors could be used to predict development of PTLD. The occurrence of EBV-associated PTLD in transplant recipients suggests that iatrogenic immunosuppression leads to a disruption of the balance between EBV persistence and immunological control of EBV that normally allows a stable lifelong infection in healthy EBV seropositive individuals.

Previous studies on the pathogenesis of PTLD have been of limited value, tending to involve low numbers of transplant recipients in prospective studies or have been retrospective studies of patients with PTLD. The majority of studies have been on paediatric transplant recipients. In contrast, this study prospectively followed all adults preparing to undergo cardiothoracic transplantation between January 1995 and November 1999 at Harefield Hospital (n=96). This allows unbiased investigation of the influence of immunosuppressive therapy on EBV infection and insights into the factors that may be involved in PTLD development. The fact that none of the 96 transplant recipients studied developed PTLD during the follow-up period of up to 3 years demonstrates the necessity for unbiased large-scale investigations into factors that cause certain patients to progress to disease.

5.2 Experimental design

The aim of the study was to prospectively follow a group of transplant recipients and monitor changes in EBV genome copy number and transcription pattern. These factors were compared with control groups and related to drug dose and clinical events.

PCRs were developed for semi-quantitative DNA analysis to determine EBV genome copy number and RT-PCRs to examine the pattern of EBV latent and lytic gene expression in PBMs from transplant recipients and controls.

Pre-transplant blood samples were available from 132 patients of which 96 were followed for up to 1110 days post-transplant (mean 415 days). In addition, blood samples were taken from 36 normal healthy EBV seropositive adults, 30 adults with acute IM and 15 transplant recipients with PTLD from Harefield Hospital and other UK transplant centres.

5.2.1 Semi-quantitative DNA PCR

A semi-quantitative PCR was developed based on detection of the Bam H1W repeat region of the EBV genome which was reproducibly capable of detecting 1 to 10 EBV genomes/10^6 cells (section 4-1). Several previously published studies have used BamH1W sequences as targets for semi-quantitative PCR (Saito *et al* 1989, Knecht *et al* 1991, Snijdjers *et al* 1990, Wagner *et al* 1992, Savoie *et al* 1994, Laroche *et al* 1995) and similar assays have been developed to detect EBNA1 (Telenti *et al* 1990) and EBNA2-encoding sequences (Sixby *et al* 1989). Other approaches for quantitative PCR were considered at the outset of this study, based on the method developed by Fox *et al* (1992) to quantify CMV DNA. In this assay the test sample is spiked with known quantity of plasmid DNA containing a sequence identical to the viral target apart from a few base changes engineered to produce a unique restriction site. This allows quantitation of target DNA through comparison of the proportion of amplified sample and control amplimers following digestion and electrophoresis of PCR products. The advantage of this method is that the control DNA is amplified in the same tube as the target sequence providing an internal standard which eliminates error due to inter-tube variation in amplification efficiency. However it was decided to continue with the semi-quantitative BamW PCR approach to avoid post-amplification processing which would greatly increase the time taken to analyze large numbers of samples. The Bam W method proved simple and reliable in providing comparison of EBV copy number between groups of subjects and in multiple samples from individual patients. Since the study began quantitative-competitive and real-time PCR techniques to quantify EBV copy number have been developed, such as those reported by Rowe *et al* (1997) and Kimura *et al* (1999). Real-time PCR methods utilizing fluorescent-tagged primers to

directly follow the production of amplimers are faster than the semi-quantitative or quantitative-competitive approach, and may prove to be an ideal method for accurately analyzing large numbers of samples. However, the start-up costs of purchasing equipment dedicated to this technique are prohibitive for research laboratories which are not routinely screening high numbers of samples. The diversity of approaches for quantitation of EBV and other herpesviruses makes comparison of data between research groups problematic, and it is therefore essential that methods should be standardized to allow meaningful assessment of the significance of virus DNA quantitation in a clinical or research setting.

5.2.2 RT-PCR

To assess EBV transcription in controls and transplant recipients, it was vital to select a panel of PCRs that gave a clear picture of the combination of genes expressed. Various primer-probe combinations were assessed to establish which assays provided the best sensitivity and reproducibility in detecting EBV transcripts.

PCR to detect gp350 mRNA, encoding the major EBV envelope glycoprotein, was chosen since its detection would indicate productive infection. EBNA3c PCR was chosen to detect latency III-like unrestricted EBNA expression since it was the most sensitive and reproducible PCR tested. Detection of EBNA3c was taken to indicate the other EBNA mRNAs were also transcribed, since the Cp or Wp promoter initiates transcription of a polycistronic message including all the EBNA genes. PCRs to detect LMP1 were included since this protein has profound effects on cell growth and is associated with most EBV positive tumours. PCRs for LMP2a and 2b were included to give a complete indication of latent gene expression in clinical samples. The PCRs used were capable of detecting latent gene mRNA (EBERs, EBNA3c, LMPs 1, 2a and 2b) from 100 B95-8 cells in a background of 1 x 10^6 EBV negative cells, and gp350 PCR detected 10 B95-8 cells/10^6 cells. Other PCRs, which were assessed but not used to test clinical samples, included detection of EBNA2 mRNA, mRNAs derived from Cp or Wp promoters (transcribing the polycistronic EBNA-encoding mRNA) (Woisetschlager *et al* 1989) and PCRs to detect EBNA1 mRNAs transcribed from alternative promoters (Schaefer *et al* 1995).

These PCRs were not used because they were insufficiently sensitive, or, in the case of PCRs to detect the various forms of EBNA1, required too many reactions to give an indication of gene expression, and were therefore too cumbersome for use with large numbers of samples.

In normal EBV seropositives and patients with IM, EBERs mRNA was the most consistently detected transcript (Tables 4-14 and 4-15). No other EBV mRNAs were detected in EBERs negative samples, and therefore this abundantly expressed transcript was used to screen samples from transplant recipients to determine whether they contained detectable levels of EBV transcripts. EBERs negative samples were eliminated from further analysis and EBERs mRNA positive samples were used for subsequent PCR analysis to detect the EBV transcripts EBNA3c, LMP2a, LMP2b, LMP1 and gp350.

5.3 EBV infection in clinical samples

A detailed model of EBV infection, persistence and reactivation has been proposed by David Thorley-Lawson and co-workers (reviewed in Thorley-Lawson & Babcock 1999) whereby EBV survives in the immunocompetent host through exploiting the normal pathways of B-cell activation and differentiation. The results of the present report are discussed in relation to this model.

5.3.1 Primary infection and infectious mononucleosis

EBV is first thought to infect naïve B-cells in the oropharyngeal submucosal epithelium. EBV latent transcription then occurs, promoting proliferation of latently infected B-lymphoblasts mirroring infection of resting B-cells with EBV *in vitro*.

Initiation of EBV transcription in newly infected B-cells is thought to occur through induction of the complement induced B-cell activation pathway by gp350/220 binding to the C3d complement receptor CD21 on the cell surface. The first EBV promoter to be activated is Wp, which transcribes the EBNA complex of genes, leading to B-cell activation and proliferation (Woisetschlager *et al* 1990). The mechanism and phenotype of B-cell activation induced by EBV gene products closely resembles that of normal B-cells that become blasts after encountering

antigen with T-cell help. Unlike normal B-cell activation by antigen however, EBV infected lymphoblasts are blocked in their differentiation and continue to proliferate.

5.3.1.1 EBV gene expression and virus copy number in patients with symptomatic primary EBV infection (infectious mononucleosis (IM))

Single blood samples from 30 adults with acute IM were used to establish the EBV load and pattern of EBV transcription in peripheral blood during this self-limiting EBV-associated lymphoproliferative disorder.

EBV load in IM was significantly higher than in normal EBV seropositive donors ($p=0.001$) with levels up to 1×10^6 genomes/10^6 PBMs (median 1×10^4 genomes/10^6 PBMs) (Table 4-10). Previous reports have identified correlations between progression of primary infection and levels of EBV in peripheral blood, since EBV copy number reduces during convalescence following IM (Rocci *et al* 1977, Laroche *et al* 1995, Yamamoto *et al* 1995, Kimura *et al* 1999). However, no significant correlation was found between reported duration of symptoms before presentation (indicating the time since primary infection) and EBV load in this group ($p=0.7$). Since follow-up samples were not available it is not possible to draw conclusions about how EBV load changes during establishment of persistent infection in this patient group.

RT-PCR analysis performed on PBM RNA samples from 28 patients with acute IM demonstrated that during primary EBV infection, infected cells express EBV latent and lytic genes which are not detectable during persistent infection (Table 4-15). Unrestricted latent gene expression was detected in 6 patients (detection of EBNA3c mRNA), LMP1 was detected in 1 patient and lytic replication detected in 9 patients (32%). There was a strong correlation between short duration of symptoms of IM and unrestricted latent gene expression ($p=0.031$), indicating that early in IM cells expressing these antigens are able to persist and proliferate, establishing a persistent infection before establishment of functional immunity to EBV.

Niedobitek *et al* (1997b) assessed EBV gene expression in lymphoreticular biopsies from IM patients directly using immunocytochemistry and *in-situ* hybridization. The combinations of EBV genes expressed in individual cells did not necessarily correspond to the accepted latency I, II, III models of EBV transcription. A diverse population of EBV infected cells may be in circulation during IM, with different combinations of latent and lytic transcription. The various combinations of EBV transcripts are therefore not necessarily expressed together in individual cells, and care should be taken in interpreting RT-PCR results from what may be a mixed population of EBV infected cells in the blood. Detection of each EBV transcript indicates expression in some of the population of cells, and assumptions should not be made as to associated expression of other transcripts in individual cells.

The occurrence of EBV replication during IM is supported by a report by Prang *et al* (1997) in which late lytic transcripts were detected in B-cells from 25% of IM patients. EBV DNA has also been detected in the serum or plasma of patients with IM, suggesting lytic replication and release of infectious virions into the blood (Gan *et al* 1994, Laroche *et al* 1995, Yamamoto *et al* 1995). Detection of EBV DNA in serum could occur through release of latent EBV genomes by cell lysis, resulting from immunologically mediated lysis *in situ* or *ex vivo* processing of samples. High levels of EBV shedding into saliva have also been reported during acute IM, therefore it appears that some lymphoblasts enter the lytic cycle and produce infectious virus particles in the peripheral blood and oropharynx during acute IM.

5.3.1.2 Establishment of persistent infection

Despite development of CTL responses against the highly immunogenic latent proteins expressed by EBV infected lymphoblasts, EBV establishes a persistent infection. It is proposed that some EBV infected B-lymphoblasts differentiate to become memory B-cells, which are detectable in healthy EBV seropositives (Miyashita *et al* 1997). The lymphoblasts detected in patients with IM therefore represent a transient stage that is required for expansion of the population of latently infected cells in the body through lymphoblastoid cell proliferation and infection of naïve cells following lytic replication.

Differentiation of EBV infected lymphoblasts into resting memory B-cells is essential for the virus to maintain a persistent infection in the face of immunological responses against latent antigens. EBV infected B-blasts are proposed to enter the lymph node where they proliferate in the same way as centroblasts in a germinal centre reaction. For infected B-cells to differentiate and become memory cells, EBNA gene expression must be switched off, since these growth-promoting proteins induce the activated B-cell phenotype and drive proliferation. Indeed if EBNA2 expression is switched off in the presence of activated c-myc, expression of the latent proteins stops and the cells differentiate and acquire the non-proliferative centrocytic phenotype (Polack *et al* 1996). Thorley-Lawson & Babcock (1999) report that EBNA2 negative, LMP1 and LMP2a and b positive centrocytes occur in the tonsils of healthy donors (Thorley-Lawson & Babcock unpublished observations) and propose that these cells could be rescued from apoptosis in lymph nodes by LMP2a and LMP1, and differentiate into resting memory B-cells. The mechanism of this LMP2a and LMP1 driven differentiation is thought to be another example of EBV utilizing the normal behaviour of antigen activated B-cells.

LMP2a contains an ITAM motif which is also present on the B-cell receptor molecule and which provides signaling when the receptor binds antigen. The LMPa ITAM motif binds src tyrosine kinases and may provide the equivalent of cell-survival signaling provided by antigen binding the B-cell receptor, thus rescuing the activated B-blast from apoptosis (Lam *et al* 1997). LMP1 acts like a constitutively active CD40 molecule, providing to an EBV infected memory B-cell the equivalent of T-helper cell signaling. In this theory LMP1 and LMP2a together can provide the signals necessary to rescue EBV-infected centroblasts from apoptosis, allowing them to become plasma cells or memory cells. These cells undergo apoptosis unless they encounter CD40 plus antigen and specific cytokines. Under these conditions the cells undergo terminal differentiation to form plasma cells. Ultimately prolonged CD40 signaling pushes remaining B-cells to form memory B-cells (Arpin *et al* 1995); the cell type in which EBV is exclusively detected in the peripheral blood (Miyashita *et al* 1997).

RT-PCR analysis of EBV gene expression in healthy EBV seropositives consistently revealed restricted latent gene expression. Neither EBNA3c- nor gp350-encoding mRNAs were detected in any normal EBV seropositives. These findings are consistent with previous reports although there is no definitive description of the pattern of EBV gene expression in persistent infection. Prang *et al* (1997) found that although transcripts encoding the lytic transactivator BZLF1 were detected in 77% of subjects there was no detectable late lytic transcription. Therefore despite initiation of lytic replication in normal EBV carriers, this may not lead to virion production, suggesting immunological elimination of cells undergoing productive infection in the peripheral blood. Despite the proposed role of LMP1 in establishing persistent infection LMP1-encoding mRNA was not detected in PBMs from normal healthy individuals in this study, and has not been reported elsewhere. LMP1 mRNA may be rapidly degraded following differentiation of lymphoblasts into memory cells, and can therefore not be detected in the circulation.

LMP2a is the only consistently detected EBV transcript in healthy donors (this report, Miyashita *et al* 1995 & 1997, Babcock *et al* 1998, Tierney *et al* 1994, Qu & Rowe 1992). Miyashita *et al* (1997) found that over 90% of EBV infected cells in normal donors were resting (CD23-ve) B-lymphocytes, and LMP2a mRNA was the only EBV transcript detected in this population. The role of LMP2a in maintaining persistent infection could be in providing B cell receptor signaling to encourage survival of the resting memory B-cell, mimicking the uninfected memory cells requirement for low levels of antigen for survival (Gray & Skarvall 1988). LMP2a may also be necessary for maintenance of viral latency and evasion of immunosurveillance through repression of reactivation (Miller *et al* 1995). LMP2a is a target for CTL (Khanna *et al* 1995) although the infected cells do not express the costimulatory molecule B7 (Miyashita *et al* 1995) and are therefore protected from eliciting a memory CTL response. Secreted LMP2a protein could act as a CTL epitope, but the persistence of cells expressing LMP2a mRNA suggests that the protein may not be expressed at sufficiently high levels to elicit a response. Thorley-Lawson suggests that EBV may in fact be transcriptionally silent in memory B-cells

and that LMP2a mRNA in circulating B-cells is residual from differentiation of reactivated memory cells or reinfection of naive B-cells in lymphoid tissue.

In this study LMP2b transcripts were detected together with LMP2a in 3/36 normal donors. LMP2b has not been reported previously in normal donors, but this may reflect the low numbers of samples tested in other studies (4 in the case of Qu & Rowe 1992, 6 in the case of Tierney *et al* 1994 and 2 in the case of Miyashita *et al* 1997). LMP2b interacts with LMP2a molecules in the cell membrane, modulating the activity of LMP2a, which may be important in maintaining latency (Longnecker & Miller 1996).

5.3.1.3 Maintenance of persistent infection and virus reactivation

In normal healthy donors, levels of EBV detectable in the peripheral blood remain low (<1 to 200 genomes/ 10^6 PBMs) and relatively constant over several years (this report, Wagner *et al* 1992, Laroche *et al* 1995, Kenagy *et al* 1995, Gan *et al* 1994, Khan *et al* 1996, Haque *et al* 1997, Rowe *et al* 1997, Maeda *et al* 1999). For EBV infection in an individual to be maintained the population of infected cells must be regenerated to restore losses through cell death or immunological elimination of infected cells. Also virus particles must be produced to enable infection of new hosts.

Thorley-Lawson & Babcock (1999) propose that recirculating EBV-infected memory B-cells migrate to mucosal lymphoid tissue and undergo activation through normal B-cell activation pathways (Anagnostopoulos *et al* 1995, Casamayor *et al* 1995). It is proposed that IL10 and TGFβ signaling cause switching to IgA expression, homing cells to mucosal epithelium (Defrance *et al* 1992). TGFβ has also been shown to induce viral reactivation *in vitro* (Di Renzo *et al* 1994). This induces memory B-cells to undergo terminal differentiation to become plasma cells and this in turn is thought to initiate EBV lytic replication, producing infectious virus particles which are released into the oropharynx (Anagnostopoulos *et al* 1995). Released virus could infect other naïve B-cells in the region which become proliferating lymphoblasts. The CTL response against EBV latent antigens expressed by lymphoblastoid cells would eliminate the majority if these cells but some could

evade the CTL response by entering lymph nodes and repeating the cycle, and differentiate into memory cells to repopulate the pool of latently infected cells in the circulation.

In healthy individuals, this balance maintains a low-level latent infection without detectable lymphoblasts in the circulation. EBV persistence therefore involves two stages: firstly, low levels of tightly latent (or transcriptionally silent) infected resting memory B-cells which provide the reservoir of latent EBV infection, and secondly, the periodic activation of latent memory B-cells in the mucosal lymphoid tissue to release infectious virus. Latently infected memory cells are tolerated since they are at low levels, but the CTL response would be activated once levels increase above a certain, unknown, threshold. The CTL response is therefore a critical controlling factor in maintenance of stable persistent infection with EBV.

5.3.2 EBV infection under disrupted cellular immunity: cardiothoracic transplant recipients

Cardiothoracic transplant recipients were prospectively studied to monitor changes in EBV load and gene expression in patients with impaired cellular immunity. These individuals are at risk of developing EBV-associated PTLD which is thought to occur through an inability to eliminate proliferating lymphoblasts which are proposed to be spontaneously generated in mucosal lymphoid tissue. No large-scale prospective study has previously followed a group of transplant recipients to monitor changes in EBV copy number and EBV transcription. Our results will be discussed in relation to the only comparable report, by Babcock *et al* (1999), which studied 28 liver and kidney transplant recipients at a single time point and investigated EBV gene expression in 4 of these. A recent report investigated the predictive value of EBV quantitation combined with RT-PCR for detection of the immediate early transcript BZLF1 in paediatric liver transplant recipients (Vajro *et al* 2000). BZLF1 mRNA was detected in 70% of 44 patients. The parameters were not predictive of PTLD when considered alone.

5.3.2.1 Pre-transplant

EBV transcription was equivalent to that in normal EBV carriers in the majority of pre-transplant samples (83%); that is no detectable EBV transcription or EBERs with or without detectable LMP2a and LMP2b transcripts. In 13 patients (17%), additional transcripts were detected (Table 4-17b). There was no apparent association between patients' underlying disease, age or sex and detection of additional EBV transcripts to those detected in normal EBV carriers. CTL activity in patients awaiting transplantation is significantly reduced compared with normal EBV positive donors (Haque *et al* 1997). However, in the results presented here there was no associated elevation in EBV genome copy number pre-transplant compared to normal carriers (p=0.542 Table 4-10). Despite underlying immunosuppression, which may allow persistence of cells expressing normally antigenic EBV latent proteins, EBV infection remains under control in patients awaiting transplantation.

5.3.2.2 Post-transplant

The cohort of transplant recipients was studied to examine whether EBV copy number and transcription pattern changed following transplantation and if so whether specific patterns predisposed to development of PTLD.

In at least one post-transplant sample from 55% of patients EBV copy number exceeded the range detected in normal EBV seropositives (interquartile range of 0-40 genomes/10^6 PBMs). The remaining 40 transplant recipients (45%) had EBV copy numbers which remained within the normal range. In 12 patients (14.3%), EBV copy number was equivalent to that of patients with PTLD (3500 to 1 x 10^6 genomes/ 10^6 PBMs) and equivalent to patients with IM (30-1.5 x 10^4) in 32 patients (40%). EBV gene expression in patient samples with EBV loads equivalent to EBV-diseases are shown in Table 4-25.

The 12 patients with EBV loads equivalent to patients with PTLD were 8 males, 4 females, aged 17-60 (mean 43 years). Two received lung transplants, 6 hearts, and 3 heart/lung. The highest detected EBV load was in a 17 year-old female with cystic fibrosis who received a heart/lung transplant, seroconverted with EBV immediately post-transplant and survived for 18 months post-transplant. The source

of the infecting EBV is not known, although EBV was not detected in DNA extracted from archival spleen tissue from the 11 year-old organ donor. EBV copy number fluctuated, but showed a steady increase, reaching a maximum of 1×10^5 genomes/ 10^6 PBMs which was detected in 3 out of 4 samples received between 1 year and 18 months post-transplant (Figure 4-20).

Patients with EBV loads equivalent to cases of IM or PTLD were analysed to investigate associations with underlying factors. There was no association with patient age ($p=0.327$), sex ($p=0.532$) or transplanted organ ($p=0.821$). The two patients with the highest detected EBV load were treated with FK506, but there was no significant association between use of FK506 and elevated EBV load ($p=0.078$). EBV copy number increased with follow-up time ($p=0.013$) and there was a highly significant association with cumulative dose of cyclosporin ($p=0.001$ by linear regression analysis. Table 4-12). This indicates that increasing cumulative exposure to immunosuppressive agents leads to increased EBV load.

Additional EBV transcripts to those found in normal EBV carriers were detected in 46 transplant recipients (51%) (Table 4-25). EBNA3c-encoding transcripts were detected post-transplant in 15 transplant recipients (16.7%) and lytic replication was also detected in 5 of these patients (5.6%). These transcripts were not detected in any of the 36 healthy EBV seropositive donors tested but were detected in PBMs from patients with PTLD and IM. Thus while unrestricted EBV latent and lytic gene expression in the peripheral blood is associated with EBV associated disease the detection of these transcripts in some healthy transplant recipients indicates that it is not on its own predictive of PTLD.

Patients in whom EBNA3c was detected were 8 males, 7 females aged 17 to 62 (mean 41.5). There was no association between detection of EBNA3c and age, sex or transplant type ($p= 0.126, 0.739, 0.297$ respectively). The only factors found to correlate with detection of EBNA3c were cumulative dose of cyclosporin ($p=0.005$) and use of FK506 ($p=0.032$) which was given to 42% of patients who tested positive for EBNA3c compared with 14% of EBNA3c negative patients. Since there is a correlation between cyclosporin dose, EBNA3c and high EBV load, this result suggests that cyclosporin is at least in part responsible for increased EBV load

through reducing CTL activity which allows persistence and proliferation of infected cells expressing EBNA3c.

Lytic replication was detected in combination with restricted latent gene expression in 23 patients (25.6%). The only factor found to correlate with detection of gp350 mRNA was use of ATG ($p=0.045$) which was given to 32% of patients testing gp350 positive, compared with 12% of gp350 negative patients. There was no association with patient age ($p=0.716$), sex ($p=0.334$) or transplant type ($p=0.075$). In 14 patients (15.6%), the only additional transcript to be detected was LMP1, but there were no associations with patient age, sex, transplant type or immunosuppressive regime.

The most significant factor associated with high EBV load was detection of gp350 ($p<0.001$). In samples in which latency III and lytic replication were detected together, EBV load was overall significantly higher than in samples expressing lytic replication with restricted latent transcription ($p=0.004$). Latency III and lytic replication therefore are most significant factors involved in increasing EBV copy number in the PBMs of transplant recipients. There is an increase in the occurrence of lytic replication in the early post-transplant period (Figure 3-21), and an increase in the detection of more unrestricted patterns of latent and lytic gene expression with increasing follow-up time.

Lymphoblastoid cells expressing unrestricted latent antigens have been reported in the peripheral blood of transplant recipients previously on only one occasion (Hornef *et al* 1995) when the cells were directly visualized by immunocytochemistry. Only one previous report has investigated EBV gene expression and EBV load in transplant recipients without PTLD (Babcock *et al* 1999). Twenty-eight solid organ transplant recipients were studied, concluding that EBV copy number increased post-transplant in two-thirds of patients due to increased numbers of latently infected resting B-lymphocytes, the same population of infected cells detected in healthy EBV carriers (Miyashita *et al* 1997). EBV latent gene expression was assessed by RT-PCR in just 4 patients. The only transcript detected was LMP2a in 1 patient, with no EBV transcripts detected in the other 3 samples. Latency III transcription (EBNA2) or EBNA1 were not detected. The authors suggest

that lytic replication is the most significant contributor to increased viral burden in these patients since lytic replication was demonstrated by detection of linear EBV genomes in 3 of 7 transplant recipients when assessed by Gardella gel electrophoresis. The present study demonstrates that unrestricted latent gene expression, associated with lymphoblastoid cell proliferation, is detected in transplant recipients without PTLD. Lytic replication was also detected in PBMs from transplant recipients in the present study, and was the most significant contributor to increased EBV load. However EBNA3c transcripts were also detected and statistically associated with high EBV load, indicating a significant contribution of lymphoblastoid cells in increased EBV load. This contrasts with the model proposed by Thorley-Lawson, in which EBV lymphoblasts are restricted to the lymphoid tissue.

5.3.3 PTLD

EBV copy number in PBMs from patients with PTLD was significantly higher than in normal EBV seropositive donors and patients with IM ($p=0.001$ in both cases (Table 4-10)). Previous studies have shown an association between high EBV load in peripheral blood and development of PTLD and suggest that this can be used as a predictive and prognostic marker for PTLD (Riddler *et al* 1994, Savoie *et al* 1994, Kenagy *et al* 1995, Rowe DT *et al* 1997, Cacciarelli *et al* 1998).

Lymphoblastoid-cell-like latent and lytic gene expression was detected in all six PTLD biopsies analysed. 10 of 11 (91%) PBM samples from patients with PTLD showed a less restricted pattern of EBV latent and lytic gene expression than that detected in normal EBV seropositives. Lytic replication was detected in 6 patients and EBNA3c detected in 5 patients. To our knowledge, EBV gene expression in the peripheral blood of patients with PTLD has not been reported previously (Rowe *et al* 2000, Vajro *et al* 2000). These unrestricted transcription patterns are also detected during symptomatic primary infection (IM) (this study Section 4.2.3 and Tierney *et al* 1994). The circulating cells in PTLD may be tumour cells that have escaped from lymphoid tissue to enter the circulation or may represent a more generalized transcriptional activation.

Previous analyses of PTLD biopsies at a single cell level by *in-situ* hybridization or immunohistochemistry have demonstrated heterogeneous EBV gene expression in tumour cells within an individual lymphoma biopsy (Rea *et al* 1994, Delecluse *et al* 1995, Oodjans *et al* 1995, Brink *et al* 1997). Brink *et al* (1997) described a correlation between tumour cell morphology and EBV gene expression, with latency I in small tumour cells, latency III in intermediate sized cells and latency II-like gene expression (LMP1 with little or no EBNA2) in large immunoblasts or Reed-Sternberg-like cells. Lytic replication was detected in all PTLD biopsies tested by Brooks and Thomas (1995) although only a small proportion of tumour cells express lytic transcripts.

The lymph node microenvironment is thought to play a crucial role in development of PTLD through provision of growth factors by T-helper cells which are abundant in tumours (Pereira *et al* 1998). The mechanism of EBV persistence proposed by Thorley-Lawson & Babcock (1999) suggests that this microenvironment may induce EBV replication and secondary infection of naïve B-cells which undergo unrestricted latent gene expression. In healthy individuals this would be a transient event, since CTLs would eliminate lymphoblastoid cells. The majority of PTLD tumours are restricted to lymph nodes which suggests that disease results from proliferation of EBV infected cells following activation by processes that occur in healthy carriers. More malignant extranodal tumours are associated with cellular genetic abnormalities which may permit aberrant cell migration (Knowles *et al* 1995). However, as shown in immunosuppressed transplant recipients and patients with PTLD, tumour-like cells appear to persist and are detectable in the peripheral blood. These results suggest that PTLD represents an extreme manifestation of loss of control of EBV replication and B-cell proliferation which is also detected in transplant recipients who do not have apparent disease. Alternatively it may be that EBV reactivation alone is insufficient to cause PTLD, and genetic events may be required for disease to occur.

The heterogeneous phenotype of cells within PTLD biopsies may reflect transitional phases in B cell development and differentiation associated with different EBV transcripts as proposed by the Thorley-Lawson model of EBV persistence and

reactivation. It is possible that similarly diverse cell phenotypes may exist in the peripheral circulation of patients with PTLD, but are not discernable by RT-PCR on a mixture of cells. In one case of PTLD a more restricted pattern of EBV gene expression was detected in the peripheral blood compared with the corresponding tumour, indicating that there may be different populations of cells, or different restrictions on phenotype, in different sites. This may indicate that even in immunosuppressed individuals with PTLD, tumour cells expressing unrestricted EBV gene transcription either do not enter the peripheral circulation or are eliminated by residual CTL activity.

5.4 Conclusions

None of the patients in this study group developed PTLD during the follow-up period of up to 3.5 years (mean follow-up time of 415 days). Since the transplant recipients studied were all adults, and mostly EBV seropositive, it might be expected that cases of PTLD would occur after a longer period post-transplant. The results of this study show that in many transplant recipients there are indications of unrestricted EBV latent and lytic gene expression equivalent to that detected in PBMs of patients with PTLD, yet have no apparent EBV associated disease. This demonstrates that although transplant recipients are severely immunosuppressed they are apparently able to retain control of persistent EBV infection. Diagnosis and prediction of risk factors for PTLD is therefore highly complex, indicating the necessity of careful monitoring of patients who show symptoms of PTLD and have high EBV load. With increasing exposure to immunosuppressive drugs there is an increase in the EBV load and detection of unrestricted latent gene expression. This correlates with previous observations that PTLD occurs 5-10 years post-transplant in seropositive adult transplant recipients (Penn *et al* 1988). CTL activity has been shown to recover following the initial 6 month period of profound immunosuppression (Haque *et al* 1997) although maintenance immunosuppression is given for life. It may be that there is gradual loss of residual EBV-specific CTL activity with increasing exposure

to immunosuppression which ultimately allows EBV infected lymphoblasts to replicate to levels which cause disease.

The detection of lymphoblastoid-cell-like EBV gene expression in peripheral blood of patients without EBV-associated disease shows that additional factors other than EBV-infection must be involved in PTLD development. These could be cellular genetic factors or provision of growth factors which could allow EBV-infected lymphoblast to accumulate to sufficiently high levels to cause apparent disease. The majority of primary tumours do not carry mutations of the tumour suppressor genes or cellular oncogenes, indicating that the tumours arise as a result of EBV-driven proliferation of normal B-lymphocytes. However, mutations have been detected in PTLD, notably associated with more aggressive disseminated tumours which often result during recurrence of the disease. This indicates a multi-step mechanism in progression of EBV infection from asymptomatic reactivation through polyclonal refractory PTLD to monoclonal malignancy.

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